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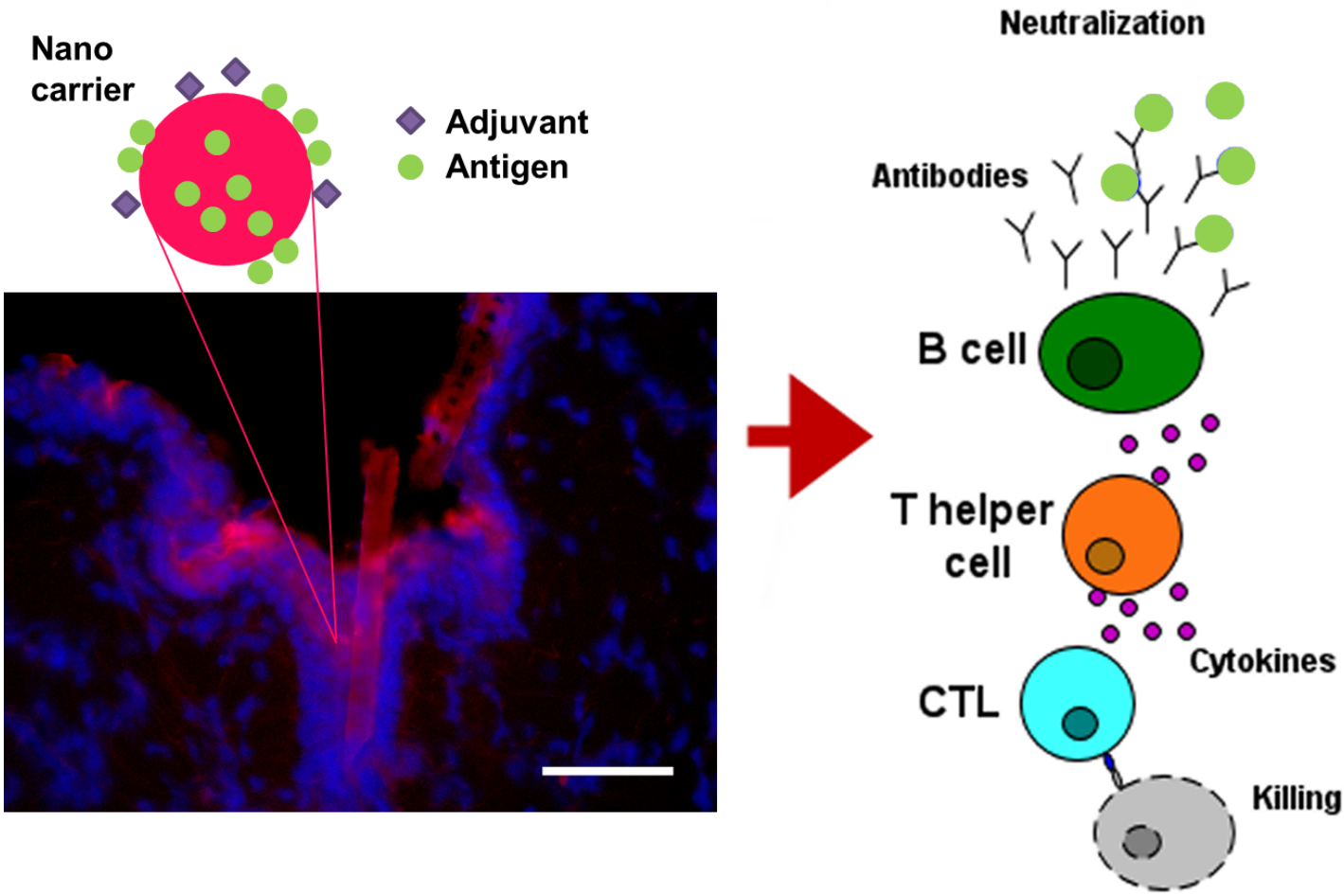

In vaccine development, activating the immune system is the major challenge. In this perspective, transcutaneous immunization (TCI) offers an attractive approach due to the presence of abundant antigen presenting cells (APCs) such as Langerhans cells and dendritic cells. In particular transfollicular vaccination aims to deliver antigens to the abundant peri-follicular APCs without compromising the SC barrier function. The present work clearly demonstrates the potential of transfollicular immunization as an approach to deliver antigens across intact skin following co-administration of nanoparticles with an adjuvant on intact skin as well as stimulating and modulating efficient humoral and cellular immune response according to the specific clinical needs.

Efficient nanoparticle-mediated needle-free transcutaneous vaccination via hair follicles requires adjuvantation

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Claus-Michael Lehr wants to declare the conflict of interest functioning as CEO of PharmBioTech GmbH, Saarbruecken, Germany. Carlos A. Guzman and Thomas Ebensen are named as inventors in a patent application covering the use of c-di-AMP as adjuvant (PCT/EP 2006010693). Other authors declare no competing financial interest.

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Abstract

Trans-follicular (TF) vaccination has recently been studied as a unique route for non-invasive transcutaneous vaccination. The present study aims to extensively characterize the immune responses triggered by TF vaccination using ovalbumin loaded chitosan-PLGA (poly lactic-co-glycolic acid) nanoparticles without skin pre-treatment to preserve skin integrity. The impact of formulation composition i.e. antigenic solution or antigen-loaded nanoparticles with or without adjuvant (bis-(3',5')-cyclic dimeric adenosine monophosphate) on immune response quality following TF immunisation was analysed and compared with immune responses obtained after tape stripping the skin. The results presented in this study confirm the potency of nanoparticle based vaccine formulations to deliver antigen across the intact skin via the follicular route, but at the same time demonstrate the necessity to include adjuvants to generate efficient antigen-specific humoral and cellular immune responses.

Keywords: Chitosan-PLGA nanoparticles; Ovalbumin; Transfollicular; Vaccination.

Background

Vaccination is one of the most promising strategies to prevent infectious diseases. Its potential therapeutic use against communicable and non-communicable (*e.g.* cancer) diseases is also attracting considerable interest. However, to meet the current challenges of vaccine delivery an easy-to-use, needle-free and non-invasive delivery system is needed.[1] In this perspective, transcutaneous immunization (TCI) offers an attractive approach for the development of highly accepted and needle-free vaccines, which are not only safe but also effective due to the presence of abundant professional antigen presenting cells (APCs), such as dendritic cells (DCs) and Langerhans cells (LCs), in different layers of the skin.

However, the main challenge for TCI is to enhance the transport of antigens across the stratum corneum (SC) barrier. To this end, reversible barrier disruption methods are often applied, such as chemical permeation enhancers, abrasion, electroporation, micro-needles, PowderJect and gene gun.[2] In contrast, TF vaccination aims to deliver antigens to the abundant peri-follicular APCs without compromising the SC barrier function.[3] Nanoparticles (NPs) have been shown to be ideal vehicles for TF delivery, since they preferentially accumulate and penetrate deeper into hair follicles than conventional formulations.[4] In our previous studies, we have shown that this holds also true in case of delivery of antigenic molecules encapsulated into NPs not only in terms of penetration of antigen in hair follicles but also quantitatively. We observed 2–3 times higher antigen amounts in the hair follicles of excised pig ear skin, when the antigen had been encapsulated in NPs as compared to antigen applied as solution.[5]

However, TF vaccination is usually performed after pre-treating the skin area by plucking hairs, waxing or cyanoacrylate (superglue) stripping.[6-8] Pre-treatment not only permeabilizes the skin by removing the upper layers of the SC, but also activates the innate immune system.[1] While both factors probably improve the immunogenicity of a topically applied vaccine, the disadvantage of the method is that permeabilization of the skin increases

considerably the risk of infections.[9] Thus, for various purposes, such as mass vaccination campaigns or vaccination of immune-compromised individuals, elderly, people with poor wound healing or young children these strategies are suboptimal.

Our previous studies investigated the potential of the TF route for the delivery of antigen using NPs without pre-treating the skin for the purpose of non-invasive TCI. As a proof of concept, we showed the successful delivery of antigen via the TF route using antigen-loaded NPs in an adoptive transfer mouse model by measuring the proliferation of antigen-specific CD4+ T cells.[5] Although the adoptive transfer model demonstrated the viability of this vaccination route, the experimental setting does not resemble a true vaccination. The proliferation of ovalbumin (OVA)-specific CD4+ T cells provides a hint on the feasibility of this approach. However, while in a normal mouse antigen-specific T cells are supposed to be in a ratio of 1 in 100,000, this ratio is artificially elevated to at least 1 in 100 by the adoptive transfer method.

Here, we extended our *in vivo* mouse studies in a normal vaccination setting to confirm the viability of the TF route, as well as to obtain deeper insights in the immune responses generated using NPs without pre-treatment of the skin. To this end, vaccination experiments were performed in conventional mice in which i) skin integrity and maintenance of the skin barrier function was monitored throughout the experiment, ii) immune responses generated by applying different formulations on tape stripped and intact skin were compared, and iii) immune responses generated by applying antigen-loaded NPs on skin without compromising the SC barrier in the presence or absence of an adjuvant were extensively characterized. The obtained results should assist in the rational designing of particulate formulations for prophylactic or therapeutic vaccination via the TF route.

Methods

Particle preparation

OVA-loaded Chitosan-PLGA (Chit-PLGA) NPs were prepared by a modified double emulsion method (more details in supplementary materials).[5] Dialkylcarbocyanine (DiD, lipophilic fluorescent dye) loaded NPs were prepared in a similar manner (from unlabelled PLGA) with the difference that 40 µl of DiD ethanolic solution was added into the organic phase.

Mice

Female C57BL/6 (H-2b) mice 6–8 weeks old were purchased from Harlan Germany. All animal experiments in this study have been performed with ethical agreement by the local government of Lower Saxony (Germany) with the No. 33.11.42502-04-017/08.

Measurement of transepidermal water loss (TEWL)

TEWL was assessed on the dorsal side of flank part of four C57BL/6 mice skin using an AquaFlux AF200 which operates with a closed measurement chamber (Biox Systems Ltd., London, UK). Measurements of TEWL were taken on day 0 (of untreated skin, serving as control reading, and again after performing depilation), on day 1 and day 2, according to the manufacturer's instructions.

Immunization protocols

Immunization was carried out on the flanks of the mice. In brief, 2 days before the immunization mice were anesthetized and hair was removed using clippers and a depilatory cream. Depilated areas were carefully inspected for cuts or skin irritation, in which case these mice were excluded from the experiment.

Female C57BL/6 mice (n = 5) were immunized on day 0, 14, 28 and 42 by applying 60 µl of different formulations containing 200 µg of LPS-free OVA topically to an area of 2.25 cm² of either intact or tape stripped skin (Table 1).

Tape stripping was performed as follows: 5 successive adhesive tapes (Tesafilm kristallklar, Tesa SE, Germany) were removed from the application area. For each stripping, a fresh piece of tape was lightly pressed onto the skin and subsequently pulled off. Directly after stripping, control or vaccine formulations were applied and allowed to dry completely. During this procedure the mice remained anesthetized to secure optimal uptake of the formulations.

In vivo localization of nanoparticles using histopathology

Histo-pathologic studies were performed on skin sites 4 h after topical application of DiD loaded Chit-PLGA NPs. Skin sites were removed and immediately embedded in O.C.T™ (Tissue-Tek®, Sakura Finetek Germany GmbH, Germany) for cryopreservation. Frozen tissues were sequentially sectioned into 6 µm slices with a Microm HM 560 cryostat (MICROM International GmbH, Walldorf, Germany). Subsequently, sections were stained with DAPI (4´,6-diamidine-2-phenylindoledihydrochloride, Roche) and analyzed with a fluorescence microscope.

Sample collection

Blood samples from immunized mice were taken from the retro-orbital complex on day −1, 13, 27, 41 and 56 (more details in supplementary materials).

Detection of antigen-specific IgG and IgG subtypes

OVA-specific antibodies in sera of individual animals were determined by ELISA, using microtiter plates coated with 100 µl/well (OVA 2 µg/ml in 0.05 M carbonate buffer, pH 9.6), as previously described (more details in supplementary materials).[10]

Measurement of cellular proliferation

Spleen and lymph node cells of vaccinated mice were obtained by mashing the organs and subsequently lysing erythrocytes for 1 min with ammonium chloride (ACK) buffer, as previously described (more details in supplementary materials).[10]

Evaluation of cytokine profiles

To quantify the cytokines and chemokines secreted by antigen-specific immune cells, cells were re-stimulated *in vitro* with different concentrations of EndoGrade® OVA (Hyglos, Germany). After 96 h of incubation, supernatants were collected and stored at −80 °C until processing. Then, the amounts of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, TNFα and IFN-γ were determined using the Mouse Th1/Th2 FlowCytomix cytokine array according to the manufacturer's instructions (eBioscience, Bender MedSytems®, USA).

Multifunctional T cells

In order to evaluate the capacity of the different vaccine formulations to stimulate OVA-specific multifunctional T cells, splenocytes from immunized mice were taken and their capacity to produce different cytokines was evaluated by flow cytometry (more details in supplementary materials).

Statistical analysis

The statistical significance of the differences between experimental groups was analyzed using one way ANOVA. Differences were considered significant at $p<0.05$ (*) and highly significant at $p<0.001$(***).

Results

Characterization of OVA-loaded Chit-PLGA NPs

The characteristics of the NPs are summarized in Table 2. The mean size of OVA-loaded Chit-PLGA NPs was ca. 180 nm and a monodisperse size distribution (PDI < 0.2). The particles carried a positive surface charge and had an entrapment efficiency (wt OVA encapsulated/wt OVA added initially) of 30.85% ± 1.08% and % loading (wt OVA encapsulated/wt polymer) 6.65% ± 0.18%. Admixing the adjuvant to the OVA-loaded NPs did not change the particle characteristics in terms of size and charge (data not shown).

TEWL Measurement

TEWL measures the skin barrier towards water and is a well-established non-invasive tool in dermatology to assess the integrity of the skin barrier *in vivo* and for testing skin irritancy.[11] Its high sensitivity allows detecting clinically invisible damage to the skin induced by various physical and chemical injuries. When skin is damaged, its barrier function is impaired resulting in higher trans-epidermal water loss. To evaluate if the skin barrier was intact on the day of immunization, *i.e*. 2 days after depilation, TEWL was measured before treatment, directly after depilation and on two subsequent days. Just 30 min after depilation, the TEWL measurement of the depilated area was twice that of normal untreated skin, thereby showing the barrier disruption via this procedure even in the absence of any visible cut or irritation of the skin. As shown in Figure. 1, the skin barrier recovered to its normal value within 2 days of the depilation procedure.

In vivo localization of nanoparticles by histopathology

Applying antigenic solutions onto intact skin usually does not result in the stimulation of immune responses as the SC forms a barrier towards penetration of foreign substances. However, NPs are preferentially taken up into hair follicles and accumulate in the upper duct

(infundibulum). Figure 2 shows a representative image illustrating the distribution of DiD (fluorescently dye) loaded Chit-PLGA NPs on the skin surface and in the hair follicle after application to mouse skin. It is apparent that the NPs accumulated in the follicle openings, cover the hair and invade into the follicular duct.

Characterization of immune responses induced after vaccination

Humoral immune responses

Serum anti-OVA IgG antibody levels obtained after TF vaccination with OVA and OVA-loaded NPs with or without co-administration of bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP) as adjuvant on intact or tape stripped skin are shown in Figure 3A. OVA alone applied onto the intact skin or tape stripped skin promotes very low IgG titer. However, stripping the skin results in significantly ($p < 0.5$) increased IgG titer for OVA-loaded NPs as compared to OVA-loaded NPs applied on intact skin. OVA + c-di-AMP applied on intact skin also promoted low IgG titer. Interestingly, OVA-loaded NPs + c-di-AMP applied on intact skin stimulated significantly increased ($p < 0.5$) OVA-specific IgG titer in comparison to all tested formulations, except OVA NPs applied onto the tape stripped skin. For mice immunized with OVA-loaded NPs + c-di-AMP, the difference in OVA-specific IgG titer after priming and the 1st boost were less distinct. However, after the 2nd and 3rd boost significantly increased titer ($p<0.001$) were obtained in comparison to all tested groups, except OVA NPs applied on tape stripped skin after 2nd boost ($p<0.05$) (Figure. 3B).

The ratio of IgG1:IgG2c is an indication on whether the stimulated cellular immune response is Th1 (IgG2c) or Th2 (IgG1) biased or balanced. All groups receiving OVA alone or OVA-loaded NPs applied either on intact skin or tape stripped skin mainly showed anti-OVA IgG1 but not IgG2c. However, admixing the adjuvant with the NPs resulted in significantly increased levels of both OVA-specific IgG1 and IgG2c when applied on intact skin (Figure. 3C).

Measurement of cellular proliferation

The stimulation of antigen-specific cellular immune responses in mice after immunizing them with different OVA formulations on intact and tape stripped skin was then analyzed. To this end, crude cell preparations (*i.e*. containing APCs, T and B cells) obtained from lymph nodes and spleens were re-stimulated *in vitro* with different concentrations of OVA for 96 h and cellular proliferation was determined by measuring the incorporation of [^{3}H] thymidine.

The strongest proliferative capacity was observed for lymphocytes derived from mice immunized with OVA-loaded NPs co-administered with c-di-AMP, as demonstrated by the stimulation index (Figure. 4). In contrast, lymphocytes of mice immunized with OVA alone or OVA-loaded NPs showed no proliferative capacity when re-stimulated with OVA. A similar pattern was observed with splenocytes (data not shown).

The cytokine secretion profiles of lymphocytes from vaccinated mice were then analyzed by cytometric bead arrays (Figure. 5). Again, the strongest cytokine production was observed in mice immunized with OVA-NPs co-administered with c-di-AMP (Figure. 5). No differences were observed in the cytokine profiles of groups receiving OVA protein via the TF route with highest levels of IL-13 followed by IL-5, and IL-10 (Th2 cell cytokines). When OVA protein was co-administered with c-di-AMP production of IL-17, IL-4 and IFNγ was also stimulated (Figure. 5). Interestingly, the same was true when analysing the profiles stimulated by OVA-NPs applied either via intact or tape stripped skin. However, strong IFNγ production was only achieved by either tape stripping the mouse skin prior to immunization with OVA-NPs or by adding c-di-AMP as adjuvant for the intact skin (Figure. 5). Thus, the necessity of breaching the skin barrier in order to elicit efficient cellular immune responses can be overcome by co-administration of OVA-NPs with c-di-AMP.

T cell responses following vaccination

Beside the magnitude of cellular responses, vaccine efficacy also depends on the quality of the stimulated antigen-specific T cell responses. There is consensus that multifunctional T cells are associated with enhanced protection against infection, likely based on a broader functional spectrum.[12] Thus, the quality of the T cell responses stimulated by different

vaccination regimes was investigated. Immunization via the TF route stimulated only single (IFNγ+) and double positive (IFNγ+/TNFα+, IFNγ+/IL-2+, IFNγ+/IL-17+) antigen-specific CD8+ T cells (Figure. 6A). Interestingly, only in mice tape stripped prior to vaccination increased numbers were observed whereby IFNγ+/IL-2+ double positive CD8+ T cells constitute the predominant subset (data not shown). However, as shown for the stimulated cytokine profiles, co-administration of c-di-AMP with OVA-NPs not only elicits a balanced Th1/Th2 response, but also resulted in the strongest stimulation of antigen-specific CD8+ responses (Figure. 6A). When analyzing the quality of the stimulated CD4+ responses, it is even more obvious that only incorporation of c-di-AMP results in efficient cellular responses (Figure. 6B). Furthermore, co-administration of OVA-NPs with c-di-AMP efficiently stimulated multifunctional double (IFNγ+/TNFα+, IFNγ+/IL-2+) positive CD4+ and CD8+ cells as well as triple positive (IFNγ+/TNFα+/IL-2+) CD4+ cells (Figure. 6B).

Discussion

It is quite evident in literature that skin is an attractive organ for immunization that can be easily manipulated for vaccination purposes. Recently, the topical delivery of antigens formulated into particulate delivery systems has evoked considerable interest. NPs have been shown to be promising carriers for TCI and for modulating the immune response depending on the site of delivery.[13] In particular, TF vaccination using NPs holds potential for non-invasive and needle-free vaccine delivery without disrupting the barrier properties of the skin. Thus, it has been shown that NPs of a size of approx. 200 nm were taken up by the LCs located around the hair follicles.[6] Moreover, TCI with antigen (gp100 protein) loaded chitosan NPs was shown to improve the survival of tumor bearing mice in comparison to antigen solution.[14] However, it remains unclear, if the particles were applied on intact or tape stripped skin.[14] Stripping results not only in mild to moderate skin disruption, but also activates the innate immune system. Mattheolabakis et al. showed the efficacy of TCI following a prime-boost protocol using OVA-loaded poly-lactic acid NPs.[16] Mice received a priming and a 1st boost immunization via the topical route, whereas the 2nd boost was

administered via the subcutaneous route.[16] Although even modest barrier disruption is immunostimulatory and results in increased immune responses, disruption of the skin barrier considerably increases the risk of infection. This is particularly important in countries with low hygiene standards and for mass vaccination campaigns in which it is impossible to pre-screen vaccines to identify those with high risk for infections. In the present study an in depth characterization of the immune responses stimulated after TF immunization using NPs as a needle-free vaccination strategy without any barrier disrupting measure is described.

For this purpose it is essential to demonstrate skin integrity and the maintenance of skin barrier function following hair trimming and depilation throughout the experiment. Interestingly, when we evaluated the skin integrity by TEWL measurement before and after depilation we found that TEWL doubled those of the normal untreated skin already 30 min after depilation. Thus, although there was no visible skin damage or irritation, the barrier function of the skin was narrowed. However, the skin seemed to recover fully within 2 days after depilation, as TEWL values returned to the baseline levels observed before depilation. Therefore, this study revealed that careful analysis of the skin barrier integrity is mandatory before applying the formulation on depilated skin. This is particularly important considering that, according to the literature formulations are often applied immediately or within 30 min of depilation.

Humoral immune responses observed after applying different vaccine formulations on intact and tape stripped skin were compared. OVA-loaded NPs applied on tape stripped skin promoted significantly higher anti-OVA IgG titer in comparison to OVA-loaded NPs applied on intact skin. This result is in agreement with the studies done by Li *et al*. showing an increase of IgG response after stripping the skin which might be due to both increased penetration and stimulation of the innate immune system by the mild barrier disruption caused by tape stripping.[19] However, co-administration of c-di-AMP with OVA-loaded NPs on intact skin promoted the highest IgG titer among all tested formulations. In contrast, only low IgG titer were observed when OVA + c-di-AMP solution was applied on intact skin, indicating a synergistic effect of NPs and adjuvant in the formulation that results in strong humoral

immune responses. This potentiated immune response may be explained by: i) enhanced delivery of antigen to hair follicles when encapsulated into NPs[5] and ii) activation of the LCs located near to hair follicles by the adjuvant. Together both mechanisms promote enhanced antigen delivery to LCs and their subsequent activation. This in turn leads to antigen processing by LCs, their migration to the draining lymph nodes, and antigen presentation to resident T cells, thereby initiating adaptive immune responses.[20]

In line with previous reports showing that OVA alone applied onto intact or tape stripped skin generates a more Th2 biased immune response, as indicated by the production of IgG1,[21] similar results were obtained here in case of OVA-loaded NPs, which also stimulated mainly the production of IgG1. Thus, in order to evaluate the impact of adjuvant not only on the strength of the OVA-specific immune responses, as indicated by increased antibody and cytokine titers, but also on the type of stimulated immune response (indicated by both the IgG1/IgG2c ratio and the production of Th1/Th2 cytokines), we co-administered c-di-AMP along with OVA solution and OVA-loaded NPs onto intact skin. c-di-AMP is known to stimulate balanced Th1/Th2 responses and cytotoxic responses when applied via the mucosal or systemic route.[10] Interestingly, when OVA protein was applied together with c-di-AMP on the intact skin, no modification of the T helper cell response was stimulated, as indicated by the observed IgG1 and Th2 cytokines dominated response. In contrast, when mice received OVA-NPs + c-di-AMP a balanced IgG1/IgG2c response (*i.e*. indicative of a balanced Th1/Th2 pattern) was stimulated. Again, only the synergistic effect of NPs and c-di-AMP results in a modification of the stimulated immune response. This is in line with reports by Mahe *et al.* showing improved uptake and translocation of nano-encapsulated antigen via the hair follicles.[6] Similarly Kahlon *et al*. observed that modification of the immune response stimulated in mice following TCI with OVA using cholera toxin as adjuvant was achieved only when animals were tape stripped prior to vaccination, *i.e.* only after damaging the skin barrier and thus enabling transcutaneous OVA/adjuvant delivery.[22]

These results were confirmed by the analysis of the cytokines produced by lymphocytes of immunized mice. Only cells derived from mice immunized with OVA-NPs + c-di-AMP

secreted significant amounts of both the Th1 cytokine IFN-γ and the Th2 cytokines IL-4, IL-5, IL-10 and IL-13, thereby reflecting a balanced Th1/Th2 response. In contrast, cells derived from all other groups secreted mainly Th2 cytokines, and showed only marginal levels of IFN-γ. The observed cytokine profiles also correlate with findings showing that Th2 biased immune responses recruit eosinophils from bone marrow and blood to the sites of inflammation.[23] Eosinophils were shown to act as antigen-presenting cells which interact with CD4+ T cells resulting in the production of IL-4, IL-5 and IL-13 by the latter.[24-26] Furthermore, the shape of T helper responses stimulated in skin diseases depends on IL-13 and IFN-γ rather than IL-5.[27] This would further explain the balanced Th1/Th2 response stimulated by TF immunization of mice with OVA-NPs + c-di-AMP observed here. Taken together, to stimulate not only strong antibody and Th2 responses following TF immunization, but also efficient Th1 and CD8+ responses, incorporation of adjuvants in the vaccination regimes is necessary to further promote cellular responses and the stimulation of balanced Th1/Th2 responses is required.

TF immunization of mice with OVA-NPs + c-di-AMP formulation stimulated not only antigen-specific antibody responses, but also CD8+ T cell responses. In line with previous reports, CD8+ responses were stronger in mice tape stripped prior to vaccination with OVA or OVA-NPs.[13] However, co-administration of c-di-AMP surpassed skin disruption and stimulated the strongest antigen-specific CD8+ responses. Furthermore, this formulation also increases the quality of the immune responses by stimulating antigen-specific multifunctional CD8+ T cells, which were shown to be more efficient in terms of killing as compared to single producers. More specifically, TF immunization via intact skin with OVA-NPs + c-di-AMP stimulated CD8+ T cells that secrete both IFNγ and TNFα and IFNγ and IL-2 (data not shown). While IL-2 is needed in order to expand T cell responses which in turn could enhance CD8+ T cell memory, IFNγ and TNFα co-producers have enhanced cytolytic activity. In addition, OVA-NPs co-administered with c-di-AMP also increased the quality of the stimulated T helper responses as indicated by the elicitation of antigen-specific multifunctional CD4+ T cells. This

is of interest, as these cells were shown to be essential, rather than CD8+ T cells, in order to protect against different pathogens.

In summary, the results presented in this study provide the proof-of concept for the potential of NP-based TF vaccination as an approach to deliver antigens across intact skin. Incorporation of an adjuvant in the formulation seems to be essential in order to generate both efficient antigen-specific humoral and cellular responses without breaching the skin barrier as well as to modulate such responses according to the specific clinical needs.

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Figure captions

Figure. 1. Determination of the integrity of the skin at different time points according to TEWL measurements. A) Normalized TEWL measurement at different time points. B) Individual TEWL measurement at different time points (n=4). Standard deviation (STD) is indicated by vertical lines

Figure. 2. Localization of DiD loaded Chit-PLGA NPs 4 h after topical application on the skin of the flank of mice. The analysis of cryosections (6 µm) showed NPs which are visible on the skin surface and penetrate inside the hair follicles. Scale bar is 50 µm.

Figure. 3. Systemic humoral immune responses stimulated in C57BL/6 mice (n = 5) after four vaccinations with different OVA-containing formulations via intact and tape stripped skin. (A) OVA-specific IgG titer in sera after immunization (n = 5). (B) Kinetic analysis of OVA-specific IgG titer in sera of immunized mice on day 13, 27, 41 and 56. The titer observed following immunization with OVA-NPs + c-di-AMP were statistically different to all other groups with $p<0.05$ (*) and $p<0.001$ (***), respectively. (C) Analysis of OVA-specific IgG subclasses stimulated in mice 12 days after the last immunization with different OVA-containing vaccine formulations. Results are expressed as log2 of the last dilution giving the double value (OD450 nm) of the background value (negative control). Standard error of mean (SEM) is indicated by vertical lines. Differences were considered significant whenever. The IgG2c titer observed following immunization with OVA-NPs + c-di-AMP was statistically different to all other groups ($p<0.05$ (*)), and the IgG1 titer was different to the control group with $p<0.05$ (*).

Figure. 4. Evaluation of the cellular responses stimulated in mice after four vaccinations with different OVA-containing formulations via intact and tape stripped skin. Lymph node cells from vaccinated animals were collected 12 days after the last immunization and restimulated

with different concentrations of OVA for 96 h. Cellular proliferation was then assessed by determination of the [^{3}H] thymidine incorporated into the DNA of replicating cells. Results are averages of quadruplicates and expressed as stimulation index (SI). The differences were considered significant whenever $p<0.01$ (**) and $p<0.001$ (***), respectively.

Figure. 5. Analysis of cytokines secreted by immune cells of vaccinated mice. Cells were collected 12 days after the last immunization and re-stimulated in triplicates with different concentrations of OVA for 96 h. Results are expressed in pg/ml. Standard error of mean (SEM) is indicated by vertical lines.

Figure. 6. T cell responses stimulated following vaccination via TF route. Cells were collected at day 14 after the last immunization and subsequently incubated for 24 h in the presence and absence of OVA. Results are expressed as difference between re- and non-restimulated % of all CD8+ and CD4+ cells, respectively, expressing IFNγ. Living cells were gated for CD3+ CD8+ and CD3+ CD4+ double positive cells, respectively. These subpopulations were further divided into mono-functional expressing only IFNγ, bi-functional expressing two cytokines (IFNγ / IL-2 or IFNγ / TNFα) and tri-functional expressing IFNγ, IL-2 and TNFα. Pie charts represent the proportion of tri- (black), bi- (dark gray) and mono-functional (light gray) cells.