Chapter 7

Summary, discussion and perspectives
Summary

The study described in this thesis first evaluated transcutaneous immunization (TCI) of mice with diphtheria toxoid (DT) and influenza haemagglutinin antigen onto microneedle array-pretreated skin. On this basis, immune modulation of various adjuvants, *e.g.* \( lpxL1 \) lipopolysaccharide (LPS), Quil A, CpG, cholera toxin (CT) was assessed when mixed with DT and co-administrated in TCI. Another approach to overcome the skin barrier and improve TCI is to formulate antigens with vesicular carriers. For this purpose, two types of DT-containing vesicle formulations were prepared and characterized. Their immunogenicity, initiated after TCI of mice onto intact or microneedle array-pretreated skin, was also investigated in this thesis.

Attempts to improve TCI started with the use of microneedle arrays. In Chapter 3, an electric impact applicator with an optimized projecting speed was employed [1]. It enabled shorter microneedle array (300 μm-long) to pierce mouse skin uniformly and reproducibly, indicated by Trypan blue staining and trans-epidermal water loss (TEWL). TCI with DT and an influenza antigen were performed using CT as an adjuvant. For DT, microneedle array pretreatment was crucial to achieve substantial IgG and toxin-neutralizing antibody titers. It resulted in a 1000-fold increase in IgG levels as compared to TCI without pretreatment. Addition of CT further enhanced the immune response to a similar level as was observed following subcutaneous injection of DT-alum. In contrast, microneedle array pretreatment showed no significant effect on TCI with influenza antigen, whereas the response was strongly improved by co-administration of CT. These results indicated that the effect of microneedle pretreatment on TCI depended on the nature of the antigen used. Therefore, the subsequent studies were focused on DT only.

As vaccines delivered into the skin target different subsets of dendritic cells (DCs) compared to conventional injections, in Chapter 4, immune modulation by various adjuvants in TCI with DT was investigated. The immune response was significantly augmented by microneedle pretreatment of the skin. The addition of an adjuvant further increased the DT-specific serum IgG response to different extents: Quil A < CpG < CT. The IgG1/IgG2a ratio of DT-specific antibodies decreased in the following sequence: plain DT, Quil A, CT and CpG. This suggested that the Th2-biased immune response induced by plain DT could be skewed towards the Th1 direction, depending on the adjuvant
used. This study demonstrated that the potency and quality of the immune response in TCI can be optimized with the use of adjuvants.

In **Chapter 5**, a surfactant-based vesicle formulation containing DT was developed and characterized, as it has been reported that elastic vesicles efficiently transport low-molecular-weight drugs across the skin [2]. The vesicles were composed of sucrose-laurate ester and sodium bistridecyl sulfosuccinate. Octa-oxyethylene laurate ester was included to increase the bilayer elasticity [3]. Formulation variables included: molar ratios of the components, DT concentration, buffer species, pH and ionic strength. The formulations were optimized for colloidal stability and DT-vesicle association. It was found that pH had a dramatic effect on DT-vesicle association; at pH 4.5 more than 70% of the protein was associated with the vesicles, whereas less than 20% was associated at pH 5.0. Hydrophobic interactions played an important role in this association and the structural integrity of DT was preserved during the preparation.

**Chapter 6** reports the combined approach of microneedle array pretreatment and the antigen-containing vesicle formulations. TCI of mice was performed by occlusive or non-occlusive application of the previously developed DT-vesicle formulation onto intact or pretreated skin. However, no improved immunogenicity of vesicular DT was observed as compared to free DT. In subsequent studies we observed that the loading of DT abolished the elasticity of the surfactant vesicles. This elasticity loss, the negative charge of the vesicles, and the fast DT dissociation from the vesicles under the neutral pH conditions in the skin were suspected for the relatively low titers. For this reason, cationic liposome formulations were developed with stable DT-liposome association at pH 7.4. The liposomes, composed of soybean phosphatidyl choline and 1, 2-dioleoyl-3- trimethylammonium-propane chloride salt (DOTAP), were prepared using high-pressure extrusion and resulted in stable formulations with sizes of about 150 nm. The physicochemical and colloidal properties, e.g. \( \zeta \)-potential, antigen association and vesicle elasticity, were characterized and optimized similarly as performed for the DT-vesicles. At pH 7.4, a high DT-loading capacity of the liposomes was observed, mainly due to electrostatic interactions. Incorporation of Span 80 increased the bilayer elasticity in the presence of DT without changing the DT-liposome association ratio. In the following TCI study, however, the immunogenicity of DT in cationic liposome formulations was not
increased compared to free DT on both intact\(^1\) and microneedle-pretreated skin\(^2\). One of the limiting factors was the lower diffusion rate through the conduits of liposomes relative to that of free DT solution. In addition, low immune-stimulatory properties of both types of vesicle formulations were observed when included in the culture medium of immature DCs.

In conclusion, application of free antigens (DT and the influenza antigen) and DT-containing vesicle formulations onto intact skin does not induce significant antibody responses. TCI with the influenza antigen is significantly improved by co-administration of adjuvants, independent of microneedle treatment. For TCI with DT, microneedle pretreatment and the use of adjuvants, but not antigen association to vesicles, enhances the immunogenicity. The potency and quality of the immune response in TCI can be further optimized by the use of adjuvants.

**Discussion and perspectives**

TCI offers four main advantages over conventional vaccination via injection when used in humans: i) potential immunological benefits, as skin contains more densely populated antigen-presenting cells (APCs) than muscles or subcutaneous tissue \([4]\); ii) safety potential, as it avoids direct contact of adjuvant with the general blood circulation \([4]\); iii) safe administration without long and sharp needles; and iv) economical/logistical advantages as it may be self-administrated. The efforts to improve TCI are focused on three perspectives: microneedle arrays, adjuvants, and vesicular carriers, as discussed below.

**Microneedle arrays**

In this thesis, solid microneedle arrays were used to pretreat the skin, creating small conduits to facilitate transcutaneous antigen diffusion. This is a relatively straightforward method, proven effective for TCI of DT. Crucial fabrication parameters include the number, length, tip shape and diameter of the microneedles. These parameters, in addition with the projecting speed, determine the uniformity and reproducibility of the piercing. What’s more, the diameter of the microneedles likely influences the closure time of the conduits and affects the efficacy of TCI. Studies, performed in humans, showed that

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\(^1\) Both occlusive and non-occlusive application

\(^2\) Occlusive application only
after treatment of the skin with a 300 µm-long microneedle array with a diameter of 200 µm, an increased TEWL can be observed up to 120 h when the treated skin site was kept occlusive [5]. It is also reported that under non-occlusive conditions the conduits are closed within 2-3 h [6]. As current TCI studies are performed in mice, an incubation time longer than 1 h is rather difficult as animals need to be anesthetized to prevent grooming. For use in humans, a patch can easily be worn for more than 24 h. Longer application time likely enhances transcutaneous antigen diffusion, increases bioavailability of vaccines; and requires lower dose of vaccines for effective immune protection.

To improve dose control and increase bioavailability of vaccines, microneedle arrays that are vaccine-coated, dissolvable or hollow are alternative approaches. For coated microneedle arrays, technical challenges include: i) the number, length and diameter of the microneedles, which should provide sufficient surface for vaccine coating; ii) a mild coating procedure, which provides a uniform layer of vaccine only on the shaft of microneedles while maintaining antigen integrity; and iii) a fast release of the coated vaccine when inserted into the skin [7, 8]. For dissolvable microneedles, both the vaccine coating and the microneedles will be dissolved and released during application. Therefore, instead of a mild coating, a mild fabrication technique is needed to preserve the structural integrity of the antigens. Vaccination using these two types of microneedle arrays may still be performed by vaccinees themselves.

The added value of hollow microneedle arrays is the precise and reliable dose control with potential dose sparing. However, the technical challenge is the leakage-free microinjection. The length, the tip shape and the opening of the microneedles need to be optimized to minimize the flow resistance [9]. Moreover, it requires a syringe or a micro pump and thus trained personnel, which will inevitably increase the complexity and cost of such a system.

**Adjuvants and safety**

New generation vaccines are often subunit proteins or peptides, which require an adjuvant to increase their immunogenicity. Cutaneous immunization targets Langerhans cells and/or dermal DCs. These are equipped with different pathogen-recognition receptor (PRR) sets from APCs resident in other tissues for their sentinel role. For this reason, the potency of antigen and the modulation properties of adjuvants often need to be re-evaluated in the
context of TCI. It is known that in vaccination via injection, the use of potent adjuvants may be associated with acute safety risks [10]. As vaccines are given to a predominant healthy population, safety is a highly emphasized issue. The risk for systemic side effects is expected to be lower for TCI than for injection. This is partially evidenced by observations in intradermal vaccination [4, 11]. This opens perspectives for TCI with broader and more powerful manners for safe yet effective immune-potentiation and -modulation. However, being a relatively new vaccination route, more safety profiles of TCI need to be built up to have a thorough comparison with vaccination via injection.

**Vesicular carriers**

Significant efforts have been made in developing antigen-containing vesicle or liposome formulations for TCI in the research described in this thesis. Although the results are not optimal yet, some conclusions can be drawn for future studies.

The vesicular carriers studied, didn't improve the immunogenicity of topically applied DT. For the rigid, DT-containing cationic liposomes, impaired immunogenicity appears to be caused by limited antigen transport through the conduits and their low immune stimulation to DCs. For the elastic vesicles, it is shown that the immunogenicity of DT on intact skin is not significantly influenced by the presence of the trans-epidermal osmotic gradient and the vesicle structure. Therefore, it appears that the transcutaneous diffusion enhancement of the vesicles, if any, is not sufficient to influence the immunogenicity outcome. For TCI with DT, ensuring sufficient transcutaneous antigen transport should be of the first concern, even though a study shows that enhanced transport may not guarantee improved immunogenicity [12].

In vaccination via injection, particulate/vesicular antigen delivery systems improve vaccination efficacy by mimicking the size and structure of natural pathogens and providing protection and stabilization to encapsulated antigens from degradation [13]. Co-encapsulation of antigen and adjuvant in the same vesicles/particles is an optimization strategy for both TCI and injection vaccination. It may provide stronger immune stimulatory properties by targeting antigen and adjuvant to the same APC [14]. In addition, by introducing certain endosomal escaping mechanisms into the liposomes/vesicles, pH sensitivity for example, cross presentation can be
promoted, which may induce stronger cytotoxic T-cell response, beneficial for anti-viral and anti-tumor immunity [15, 16].

In this study, high antigen-vesicle association was achieved by adjusting the charge of the vesicle components, ionic strength, the species and pH of the buffer. Positively charged vesicles/particles may facilitate the interactions with the negatively charged DC membrane surface and consequently increase the antigen uptake. However, they also tend to stick to other cell surfaces or intercellular proteins and block their further diffusion through the conduits. For anionic vesicles, diffusion appears to be easier. But high antigen-loading is more difficult to achieve with anionic vesicles than with cationic ones, as most to the antigens are negatively charged under physiological conditions. If ionic strength and pH different from physiological values are used for formulation preparation, characterization under both conditions should be performed.

Recently, with the clarification of functional specialization of skin DC subsets, targeted delivery of antigen may provide modulation on the immune response induced [17]. As mentioned above, co-encapsulation, surface modification, or covalent attachment of other PRR ligands to antigen-containing vesicles can be employed to target antigens to a specific skin DC subset. This requires more intensive characterization of the formulations and needs to be tuned for each individual antigen. Cell lines, better resembling the targeted skin DC subset, or immune active skin substitutes containing targeted DC subsets, may serve to evaluate the immune-stimulatory properties of the novel transcutaneous vaccines in vitro [12]. In vivo studies of the selected formulations may start with intradermal injection for a proof of principle test on their immunogenicity in TCI. In case of positive results, a combination with a proper skin barrier disruption method, i.e. type of microneedle arrays and application time, will further optimize their potency after topical application.

**Perspectives**

Microneedle array-mediated TCI of mice with CT-adjuvanted DT can induce immune protection as high as that from subcutaneous injection of DT-alum. A dose twenty times higher than that for injection was used to ensure sufficient diffusion in this study, although only a small fraction entered the viable skin layer. Further optimization, e.g. using targeted antigen delivery carriers, more potent adjuvants with desired modulation properties, certain type of microneedle devices or skin barrier disruption methods, together with longer application time, will certainly decrease the required dose of DT. This will
essentially enable TCI to challenge injection as a superior vaccine administration.

In this study, the skin showed diverse responses upon contact with different types of antigens *in vivo*, for example, DT vs. CT when applied on intact skin or DT vs. influenza haemagglutinin when applied on microneedle-pretreated skin. It appears that TCI need to be optimized for each individual antigen. The field will benefit from a direct comparison study performing microneedle array-aided TCI with antigens of different categories, *e.g.* different charge, size, formation in solution and origin etc.

Continuous advances in understanding the immune system, especially the immune functions of the skin, will facilitate more rational design and development of transcutaneous vaccines. Vaccination will continue to be the most effective tool in controlling infectious diseases, whereas TCI will dramatically improve vaccination practice in developing countries, in cases of mass vaccination campaigns and in counteracting bio-terrorism.
Summary, discussion and perspectives

References
