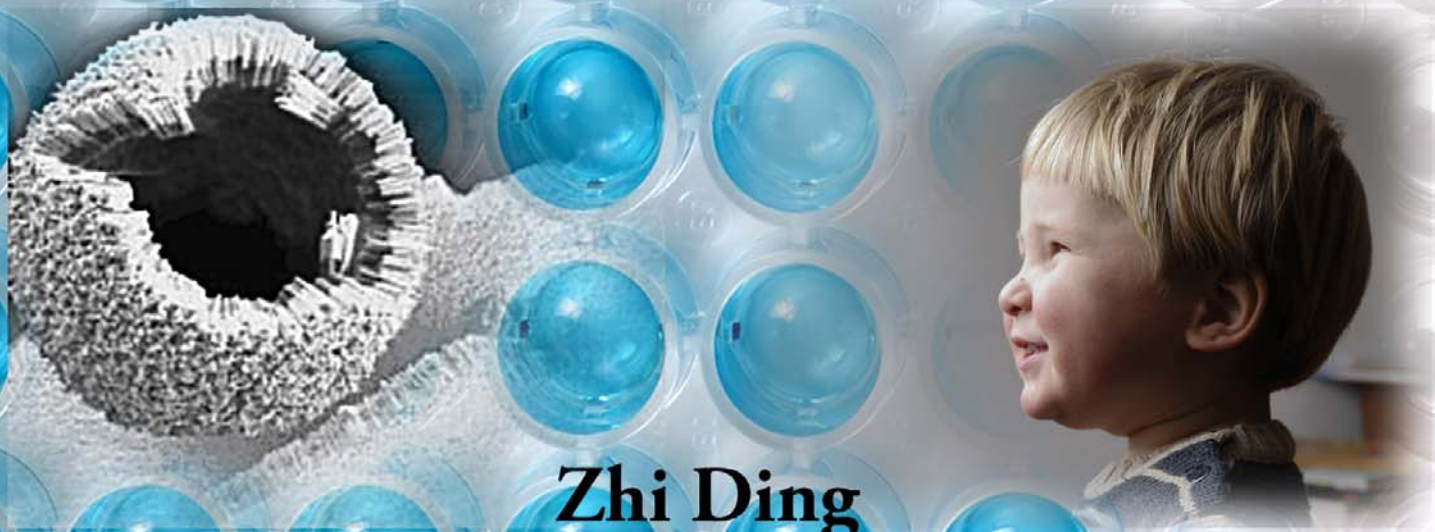
Transcutaneous subunit vaccine delivery

A combined approach of vesicle formulations and microneedle arrays



Zhi Ding

Stellingen

Behorende bij het proefschrift

Transcutaneous subunit vaccine delivery

A combined approach of vesicle formulations and microneedle arrays

1. Transcutaneous immunization of mice with cholera toxin-adjuvanted diphtheria toxoid can induce comparable immune protection as subcutaneously injected alum-adsorbed diphtheria toxoid.

   ----This thesis

2. Elastic vesicles enhance the transport of small molecules across the skin, but do not improve the immunogenicity of topically applied diphtheria toxoid.

   ----This thesis

3. The osmotic gradient in the *stratum corneum* does not play a central role as a driving force for the diffusion of topically applied antigens in elastic vesicle formulations.

   ----This thesis

4. Besides being presented by migratory skin dendritic cells, soluble antigen, when applied topically, can directly diffuse into the draining lymph nodes through lymphatics and reach the lymph node-resident dendritic cells.

   ----Itano A, *et al*., Immunity 19, 47-57, 2003

5. Joint efforts from immunologists, vaccinologists, pharmaceutical scientists, and (fine) mechanical engineers should ensure further improvement of transcutaneous immunization and essentially revolutionize the current vaccination practice.

   ----This thesis

6. The field of vaccine delivery would benefit from more direct head-to-head comparisons between administration routes in which the same doses of vaccine and dosing regimens were employed.

   ----Mikszta JA, *et al.,* Expert Rev. Vaccines 7, 1329-1339, 2008

7. Vaccine adjuvants straddle a fine line between tissue toxicity and efficacy.

   ----Wilson-Welder JH, *et al.,* J Pharm Sci 98 (4), 1278-1316, 2008

8. Considerable activity in the field of dendritic cell targeting is expected, as it has the potential of yielding a wealth of vaccines, possibly the first vaccines generated by immunologists.

   ----Ueno H *et al.*, Immunol Rev. 219:118-142, 2007

9. The formulation of the antigen and route of delivery can affect the Th1/Th2 bias of the immune response, and the type of immune response that will be protective varies with the disease in question.

   ----Wilson-Welder JH, *et al.*, J Pharm Sci 98 (4), 1278-1316, 2008

10. Struggle is nature's way of strengthening it.

    ----Locke in *Lost* Season 1. Episode 7

11. A trip of a thousand miles starts in front of your feet.

----Li Er in *Lao Zi*

12. For learning Dutch, the time that you've been staying in the Netherlands is irrelevant; what matters is the time you've spent trying to speak it.

13. Descriptions of modern China are usually out of date by the time they are printed.

Zhi Ding, Leiden, February 2010

Transcutaneous subunit vaccine delivery

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Zhi Ding

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Zhi Ding
PhD thesis with summary in Dutch and Chinese
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Transcutaneous subunit vaccine delivery

A combined approach of vesicle formulations and microneedle arrays

Proefschrift

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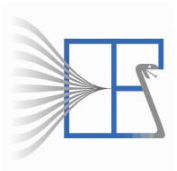

The publication of the thesis was financially supported by the Leiden/ Amsterdam Center for Drug Research and the Netherlands Vaccine Institute.

If transdermal immunization works well, vaccination practice could be revolutionized.

Stanley A. Plotkin
in "Vaccines: past, present and future"
Nature Medicine, vol 11(4), 2005

Dedicated to my wife

Table of Contents

Chapter 1

Transcutaneous immunization:

A general introduction

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Manuscript in preparation

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1. Introduction

Over the last two centuries, vaccination has been one of the most successful medical interventions in reduction of infectious diseases [1]. However, most vaccines are administrated by injection, which requires syringes, needles, and trained personnel. Injection can be painful and causes stress, especially in children. For pediatric vaccination programs, poor compliance is one of the reasons for incomplete vaccination coverage, which impairs herd immunity and may lead to unnecessary death from vaccine-preventable diseases. The needs for effective as well as non-invasive vaccine administration have boosted the research on nasal [2], transcutaneous [3], oral [4] and pulmonary delivery of vaccines [5].

The transcutaneous route is particularly attractive because the skin is highly accessible with unique immunological characteristics. It has been known for a long time that an effective immune response can be induced *via* the skin [3]. One successful example of transcutaneous vaccination is scarification in the case of smallpox immunization in humans [6]. The presence of professional antigen-presenting cells (APCs) in the epidermis and dermis mediates the cutaneous immunization [7]. Another primary reason for considering the transcutaneous route is the potential for safe immune stimulation, as it avoids the direct contact of potent (even slightly toxic) adjuvants with the general blood circulation [8]. However, the uppermost layer of the skin, the *stratum corneum*, acts as a barrier for diffusion and therefore a major obstacle to transcutaneous vaccine delivery. Currently, the main challenges for cutaneous immunization are: i) to enhance the transport of antigens across the skin barrier and ii) to improve the immunogenicity of topically applied vaccines.

In this chapter, approaches for improving transcutaneous immunization (TCI), *e.g.* vaccination through intact or pretreated skin, will be reviewed. This chapter starts with a brief introduction to vaccines and adjuvants, followed by a description of the barrier and immunological functions of the skin. The second part includes a description of innate and adaptive immune responses upon contact with an antigen and the function of skin dendritic cells (DCs). The third part summarizes the experimental approaches of enhancing transcutaneous antigen delivery and improving the immunogenicity of vaccine formulations. Finally, some safety concerns and concluding remarks are provided. For clarity, some terms used in this review are defined in Table I.

Table I. Terms used in this chapter

Term	Interpretation
Adjuvant	Substance that enhances the immunogenicity of an antigen
Cutaneous immunization	Both intradermal and transcutaneous immunization
Particle elasticity	The ability of vesicles to deform and pass through openings smaller than their actual size
Intradermal immunization	Antigen delivery into the dermis *via* a syringe and needle
Transcutaneous immunization	Antigen delivery into the epidermis and dermis through intact or pretreated skin
Microneedles	Needles shorter than 1 mm

2. Vaccines and adjuvants

Vaccines can be defined as antigen formulations that induce specific, non-toxic and long-lasting immune responses to prevent or treat disease [9]. Traditional vaccines are designed to mimic the immune response that would otherwise be induced by an active infection, thereby avoiding the undesirable consequences of the disease [10]. New vaccines can also trigger or enhance immune responses for therapeutic purposes, *e.g.* anti-cancer vaccines. However, the focus of this chapter will be on vaccines that prevent infectious diseases.

To be effective, a vaccine must contain some parts of the disease-causing agent, *e.g.* bacterium, virus, or toxin, or a substance derived from it, *e.g.* a recombinant protein or a synthetic peptide, and it may include one or more adjuvants. Antigens in the vaccine formulations are recognized, taken up and processed by APCs and subsequently presented to T lymphocytes (generating antigen-specific T cells). Vaccination regimens generally employ prime-boost strategies. Repeated administration of the same antigen induces stronger activation of effector cells (immunoglobulin-producing B cells (plasma cells), cytotoxic T cells and helper T cells, Th), and also a small population of memory B and T cells. These memory cells provide a faster and stronger secondary immune response to the same antigen upon subsequent exposure, *e.g.* in the form of an infection by a pathogen carrying this specific antigen [11, 12]. The primary mechanism of protection after vaccination is mediated by the generation of neutralizing antibodies and/or the induction of

cell-mediated immunity depending on the disease in question [13]. Currently available vaccines can be classified into three categories: modified live, inactivated and subunit vaccines.

Vaccines containing modified live organisms, such as the Sabin oral polio vaccine, induce the most potent and long-lasting immune response. They generally require the fewest number of inoculations, do not need adjuvants and are very effective at inducing both cellular and humoral immunity [14]. The largest drawback is their possible replication in immune compromised vaccinees. Moreover, there is a risk that an attenuated strain reverts to a virulent one. In this way, severe side effects may occur.

Inactivated vaccines comprise the whole organism that has been killed by treatment with heat or chemicals. Examples are the typhoid and cholera oral vaccine and the injectable hepatitis A virus. They are potent inducers of humoral immunity and possess a longer shelf life than live vaccines. However, the degree of cell-mediated immunity induced can be weak. Although safer than modified live vaccines, inactivated vaccines are highly reactogenic and associated with side effects such as high fever accompanied by severe pain, redness and swelling at the injection site.

Subunit vaccines, including DNA vaccines, contain only a portion of the organism or the gene coding for it. Being free of reactogenic agents, subunit vaccines are very safe. Toxoids, inactivated bacterial toxins such as diphtheria toxoid (DT) and tetanus toxoid (TT), are the first and very successful subunit vaccines employed for human use [15]. These toxoids are adjuvanted with alum to improve their immunogenicity. The newly developed, often recombinant, subunit vaccines mostly are poorly immunogenic, and generally require to be formulated with adjuvants.

Adjuvants are substances that accelerate, prolong or enhance antigen-specific immune responses when used in combination with vaccine antigens. Adjuvants generally demonstrate their features due to one or several of the following mechanisms:

i). provide a “depot” for the antigen, creating an antigenic reservoir for a prolonged delivery;
ii). facilitate targeting of the antigen to APCs and/or enhance phagocytosis;
iii). enhance and modulate the type of immune response induced by the antigen alone [16-19];
iv). provide a danger signal from damaged or stressed cells that the immune

system needs in order to respond to the antigen as it would during an active infection [11].

Colloidal aluminum hydroxide and aluminum phosphate, commonly referred to as alum, have been the most widely used adjuvants since 1926. Until now, alum is the only adjuvant approved (in fact, tolerated) by FDA for use in humans. It is the standard benchmark to which the efficiency of other adjuvants is usually compared [20, 21]. The mechanism of adjuvanticity of alum has long been thought to be providing an antigenic depot at the site of injection. Recent studies have shown that alum may also act *via* a different mechanism, as the alum crystals activate directly an intracellular innate immune response system called the Nalp3 (NACHT-LRR-PYD-containing protein [22]) inflammasome, or indirectly through release of the endogenous danger signal uric acid [23, 24]. While alum is effective at inducing strong humoral immunity, alum-based vaccines generally fail to induce cell-mediated immunity [25].

Most vaccines are delivered intramuscularly or subcutaneously. This may partially be due to the widespread availability of needles and syringes by which these tissues are easily accessible. There is, however, hardly any compelling evidence suggesting that they are ideal tissues for vaccination from an immunological point of view [3]. The skin is known to be a potent immune stimulatory tissue, but its full potential for vaccination has not yet been exploited.

3. Immunological function of the skin

3.1. Skin structure

The skin is the largest organ of the human body. It represents the outermost physical barrier between the body and the surrounding environment. It protects us against external mechanical impacts, ultraviolet radiation, dehydration, and microorganisms. The skin consists of three main layers: epidermis, dermis, and subcutaneous fat tissue (Fig. 1). The epidermis is the outermost layer of the skin. The human epidermis varies in thickness from 50 to 150 µm. It can be divided into four layers, which are, from outside to inside: *stratum corneum, stratum granulosum, stratum spinosum,* and *stratum basale*. The barrier function of the skin is located in the *stratum corneum*. This layer consists of rigid, desmosome-linked epithelial cells, known as corneocytes, embedded in a highly organized lamellar structure formed by intercellular

lipids. The unique arrangement of this layer (15-20 µm thick in human) results in a practically impermeable barrier which reduces the passage of molecules, especially those larger than 500 Da [26]. Underneath the *stratum corneum* resides the viable epidermis, which consists of three layers; *stratum granulosum, stratum spinosum* and *stratum basale*. The main cell type in the viable epidermis is the keratinocyte. However, melanocytes, Merkel cells and Langerhans cells (LCs), although less abundantly present, also play important roles in the functioning of the viable epidermis.

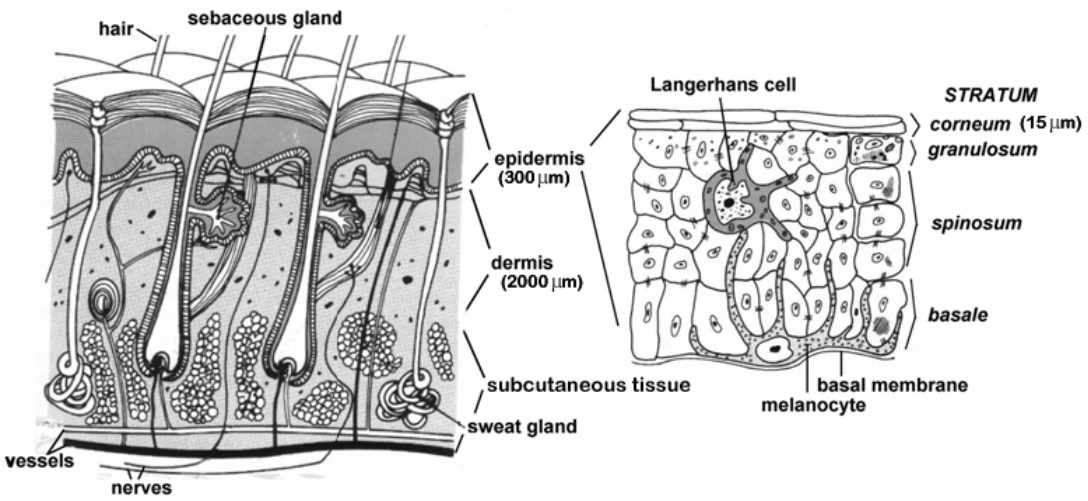

Figure 1. Structure of the skin. The skin consists of three main layers: epidermis, dermis, and subcutaneous fat tissue. The barrier function of the skin is located in the uppermost layer, the *stratum corneum*. LCs in the epidermis and the dermal DCs in the dermis are the main APCs in the skin and the targets of TCI. Image adapted from Watt [27].

Underneath the viable epidermis is the dermis. The important cell classes in the dermis are fibroblasts, mast cells, and dermal DCs (dDCs). The dermis also contains blood vessels, lymph vessels, and nerves. This skin layer is the major site of cellular and fluid exchanges between the skin and the blood and lymphatic networks. The rich blood supply of the dermis plays a role in body temperature regulation, immune responses and pain- and pressure-regulating mechanisms [28].

Beneath the dermis lays the subcutaneous fat tissue. This is an assembly of adipocytes linked by collagen fibers. It not only forms a thermal barrier, but also functions as an energy storage and a mechanical cushion for the body [29]. Appendages such as sweat glands, pilosebaceous units, and hair follicles are structures penetrating the skin and originate either from the

dermis or the subcutaneous fat tissue. These appendages form important discontinuities of the skin [28].

Besides the barrier function, the skin also has important immunological functions due to the presence of the skin-associated lymphoid tissue (SALT) [30, 31]. The SALT is constituted by APCs, such as LCs and dDCs, together with keratinocytes, mast cells, subsets of T lymphocytes and the skin lymph nodes. Although considerable amounts of microbes are covering our skin, homeostasis is maintained and we stay remarkably healthy. When microbes break the skin barrier, the immune system faces a number of options: whether or not to respond, and what the right type of response is. This decision can be a matter of life and death exemplified by for instance leprosy [32]. The skin is involved in both innate and adaptive immunity. The adaptive response enables vaccination and generally becomes more effective with each successive encounter with the same antigen, whereas the innate immune mechanism provides immediate, but short-lasting defense against infections. In the following sections about the immunological functions of the skin, the human immune system is discussed, unless stated otherwise.

3.2. Innate immunity

The most important skin cells involved in the innate immune response are the skin DCs that sample the environment, process the antigens and present these to T cells. Several distinct types of DCs are present in the skin [33]. However, as this is an emerging field of research, here only the two most established types of skin DCs, LCs and dDCs, will be described. They are two types of myeloid DCs. LCs are epidermal DCs that account for only 1% of the total epidermal cell population, but cover nearly 20% of the skin surface area [34]. LCs can be distinguished from other subsets of DCs by their expression of langerin/CD207 (Fig. 2), CD1a and E-cadherin and the presence of a unique intracytoplasmic organelle, the Birbeck granule. The dDCs are characterized by DC-SIGN (DC-specific intercellular adhesion molecule-3 (ICAM-3)- grabbing non-integrin, also known as CD209), CD11b, factor XIIIa and CD14 expression [35, 36]. They are present in higher numbers than LCs in the skin. These cells are continuously produced from the hematopoietic stem cells and distributed in an immature state as antigen-capturing cells. Recently a new subset of skin DCs has been found in the skin, *i.e.* the langerin positive $CD103^+$ DCs, which are reported to be most efficient in processing viral antigens into the major histocompatibility complex class I

(MHC I) pathway, thereby activating $CD8^+$ T cells [37]. Currently, this topic is of great interest and novel vaccines targeting specific DC subsets will be designed [38].

Skin DCs, together with macrophages recruited from circulating blood, exert their sentinel role by sampling and processing potential pathogens invading the skin. Immature DCs are activated by numerous agents derived from microbes, dying cells and cells of the innate and adaptive immune system. These responses are initiated by binding of the agents to pathogen-recognition receptors (PRRs). Although PRRs are expressed on many cell types, research on PRR activation mainly focuses on DCs, because of their important role in controlling immune responses [39]. Agents that trigger these receptors are referred to as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [40]. DAMPs include the endogenous signals, *e.g.* heat-shocked proteins (HSPs) secreted or presented by other somatic cells when dying or otherwise stressed; PAMPs usually represent exogenous signals, such as the conservative motifs of microbial products [41]. Detailed information about the PRRs, especially the Toll-like receptors (TLRs), and the corresponding PAMPs and DAMPs will be discussed later.

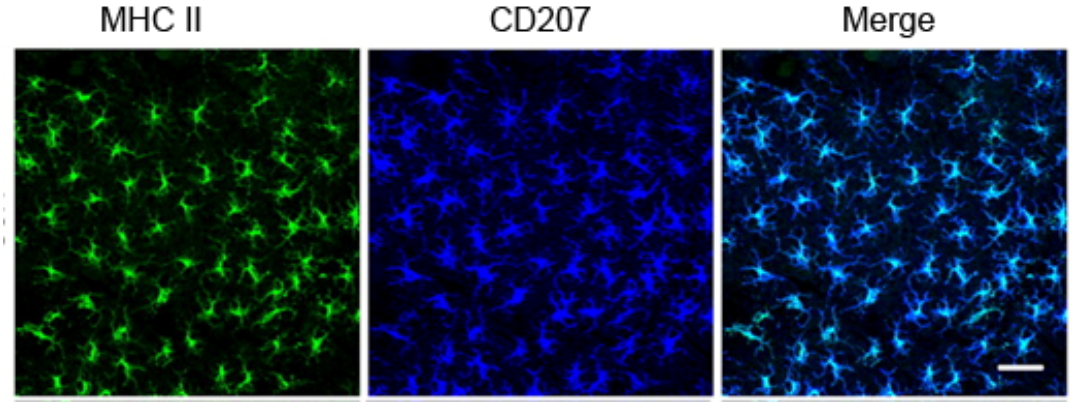

Figure 2. Epidermal LCs. Immune fluorescence staining of MHC II (green) and langerin/CD207 (blue) in epidermal sheets freshly isolated from the ear skin of a normal adult mouse (C3H, H2k). Confocal images show that all MHC II^+ cells in the epidermis express langerin. Picture adapted from Erikson *et al.* [42].

The antigen-presenting process is profoundly affected by PAMP and DAMP induced cytokines. Keratinocytes, accounting for about 90% of the total epidermal cell population, play an important role. In case of danger, *e.g.* skin barrier disruption, keratinocytes produce a wide range of cytokines such as interleukin-α (IL-1α), IL-1β, granulocyte-macrophage colony-stimulating factor

(GM-CSF) and tumor necrosis factor-α (TNF-α), which interact with DCs and help to maintain an appropriate balance between reactivity and tolerance of the immune system [43, 44]. For example, migration and maturation of LCs are initiated by pro-inflammatory cytokine IL-1β and keratinocyte-derived TNF-α [45, 46]. Besides keratinocytes, neutrophiles, macrophages, and mast cells also secrete cytokines that influence DC maturation [47, 48]. The change (differentiation) of LCs and dDCs during maturation includes increased expression of MHC molecules and co-stimulatory molecules, increased production of cytokines such as IL-1β, IL-6, IL-12, and chemokines such as CXCL1, 2, 3, 8 and CCL3-5, as well as the enhanced emigration of these cells from the skin to the paracortical area of draining lymph nodes [49, 50]. In the lymph nodes, skin-derived DCs present the processed antigens of the pathogen, together with the activation stimuli, to naïve resting T-lymphocytes surrounding them [51, 52]. This occurs in an antigen-specific fashion and results in the expansion of the respective clone(s) to mature into extremely potent immune stimulatory cells, controlling the development of adaptive immunity [53].

3.3. Pattern-recognition receptors

TLRs are important PRRs involved in host defense against a variety of pathogens. TLRs have been a central focus for immunologists and vaccinologists since they were discovered by Gay and Keith almost 20 years ago [54]. So far, ten TLR members have been identified in humans and three more in mice, each thought to selectively recognize diverse bacterial, viral stimuli or endogenous signals (Table II) [55]. TLRs can be divided into subfamilies, according to the ligands they recognize and to their cellular localization. The subfamily of TLR 1, 2, 4 and 6 recognizes lipids, whereas TLR 3, 7, 8, and 9 recognize nucleic acids [41]. Generally, TLRs that detect bacterial products other than nucleic acids (TLR1, 2, 4, 5, 6, 10 and 11) are expressed on the cell surface, whereas those detecting nucleic acids (TLR 3, 7, 8, and 9) are located intracellularly, typically on late endosomes or lysosomes. Such restricted localization might provide the mechanism by which DCs avoid spontaneous activation by self nucleic acids [56, 57]. When activated, TLRs recruit adapter molecules within the cytoplasm of cells to propagate a signal, which ultimately leads to the induction or suppression of genes that orchestrate the inflammatory response. Activation of different

TLRs regulates gateways for gene modulation and tailors the type of the induced immune responses.

Table II. TLRs and their natural ligands in humans and mice [58-63]

TLR	Ligands	Ligand location
TLR1+TLR2	Tri-acyl lipopeptides	Bacteria
TLR2+TLR6	Di-acyl lipopeptides	Mycoplasma
TLR2	Glycolipids	Bacteria
	Lipopeptide	Bacteria
	Lipoprotein	Bacteria
	Lipoteichoic acid	Bacteria
	HSP60, 70 and 90, grp96	Host cells*
	Zymosan	Fungi
TLR3	Double-stranded RNA (dsRNA)	Virus
TLR4	LPS	Gram-negative bacteria
	HSPs	Bacteria and host cells*
	Fibrinogen	Host cells*
	Fibronectin	Host cells*
	Heparan sulfate fragment	Host cells*
	Hyaluronic acid fragment	Host cells*
	β-defensin	Host cells*
TLR5	Flagellin	Bacteria
TLR7/8	Single-stranded RNA (ssRNA)	Virus
TLR9	Unmethylated CpG DNA	Bacteria
TLR10	Unknown	Unknown
TLR11 (mice only)	Profilin	Toxoplasma gondii
TLR12 (mice only)	Unknown	Unknown
TLR13 (mice only)	Unknown	Unknown

*endogenous danger signals, DAMPs

TLR expression on LCs and dDCs are different and also differs from other subtypes of DCs at mucosal surface or in the blood circulation. Epidermal LCs freshly isolated from the human skin express TLR1, 2, 3, 6 and 10 but not TLR 4 and 5. Dermal DCs do express TLR2, 4 and 5, responsible to the recognition of bacterial PAMPs. Van der Aar *et al.* proposed that the LCs' unresponsiveness to bacteria may contribute to tolerance to bacterial commensals that colonize the skin, avoiding deleterious inflammatory responses [64]. The TLR distribution on immune active skin cells (human and mouse) are presented in Table III. Some of the data are still under debate

because of different isolation methods for generating the specific types of cells. This DC heterogeneity and the differences in the epithelial microenvironment may influence the immune modulation function of certain adjuvants and thereby the choice of adjuvants for TCI.

It is generally accepted that the detection of pathogens by TLRs initiates the mobilization of the host defense against most, if not all, infectious agents. However, recent results highlight the role of other PRRs that cooperate with TLRs or compensate for TLR specialization [65]. In the absence of TLR activities, most viruses and intracellular bacteria are recognized by alternative intracellular receptor families, including nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid inducible gene based (RIG)-I-like receptors (RLRs) and c-type lectin-like receptors (CLRs) [66]. NLRs are a family of receptors recognizing intracellular microbial components, as recently reviewed [67, 68]. C-type lectins act as anchors for a large number of microbes, including viruses, bacteria, parasites, and fungi and allow their internalization. CLRs bind the carbohydrate moiety of glycoproteins and carry out multiple functions [69, 70]. In general, activation and maturation of DCs are the consequence of signal transduction within the PRR network, resulting in appropriate immunity against invading pathogens or infections.

Table III. TLR distribution in immune active skin cells [71-79]

Cell type	Human	Mouse
Keratinocytes	1-6, 9, 10	2, 4, 7, 9
LCs	1, 2, 3, 6, 10	2, 3, 4, 7, 9
dDCs	2, 4, 5	9
Myeloid DCs	1, 2, 3, 4	1, 2, 3, 4, 7, 9
Plasmacytoid DCs	7, 9	7, 9
Macrophages/Monocytes	1, 2, 4, 5, 8	3, 4, 7, 9
Mast cells	3, 9	2, 3, 4, 7, 8, 9

3.4. Adaptive immunity

Adaptive immunity provides pathogen-specific, long-lasting protection to the host. DCs are an important link between innate and adaptive immunity. They educate and stimulate B and T lymphocytes and play a central role in both stages, both cell-mediated and humoral immunity [39]. Adaptive immunity starts with the DC-T cell interaction, followed with the proliferation of T and B lymphocytes in the secondary lymphoid organs, *i.e.* spleen and lymph nodes. Both cells develop from a common lymphoid progenitor in the bone marrow. T

cells differentiate further into either $CD4^+$ helper or $CD8^+$ cytotoxic T cells. Antigen recognition by B and T lymphocytes differs from that by cells of the innate immune system in that; the latter recognize conservative motifs using PRRs, whereas B- and T-cell receptors specifically recognize a large variety of epitopes.

3.4.1. B cells

The humoral immune response is mediated by B cells. These cells recognize their cognate antigen in its native form. They recognized free (soluble) antigen in the blood or lymph using their membrane bound-IgM or IgD, which act as B cell receptors. In most cases, B cell activation, *e.g.* clonal proliferation and terminal differentiation into plasma cells, requires not only recognition of antigens, but also cytokines produced by $CD4^+$ Th cells, who are activated first after contact with APC presenting processed antigen in MHC II molecules. Special antigens, such as repeating carbohydrate epitopes from many bacteria, may also directly stimulate B cells by cross-linking the IgM antigen receptors, thereby activating them in a T cell independent manner [80]. B cells can also take up antigens and present them by MHC II to $CD4^+$ T cells. Interactions between B cells and $CD4^+$ Th cells stimulate both cell types. For example, Th2 cells are triggered to synthesis CD40L, which can bind to CD40 on B cells. As a consequence, B cells start producing large amount of antigen-specific antibodies into the blood circulation. These antibodies assist in the destruction of microbes by binding to them and making them easier targets for phagocytes and activation of the complement system.

3.4.2. $CD4^+$ T cells

In contrast to B cells, T cells only recognize their cognate antigen in a processed form, as a peptide fragment presented by an APC's MHC molecule to the T cell receptor. For complete $CD4^+$ T cell stimulation, this antigen presentation is required but not sufficient. Interaction of co-stimulatory molecules (CD80 and CD86) on DCs surface with their T cell equivalents (CD28), the secretion of stimulatory cytokines (IL-2) and a polarization signal (IL-4 and IFN-γ *etc.*) are also necessary [72, 81]. TLR recognition and activation in DCs induces the up-regulation of the activation markers CD80 and CD86, and contributes to the activation of T cells. Once a $CD4^+$ T cell is activated by a DC, it can differentiate into different types of Th cells. The

differentiation of $CD4^+$ Th cells is particularly sensitive to the type of stimulus presented to the DC. Depending on the nature of the invading pathogen, a DC can induce the differentiation of $CD4^+$ Th cells into Th1, Th2, Th17 or regulatory T cells (Treg) [82] (Fig. 3). Most bacterial and viral products, including lipopolysaccharide (LPS), bacterial DNA and dsRNA, drive the differentiation towards a Th1 functional phenotype [83, 84]. Th1 cells secrete IL-2, IL-12, IFN-γ and TNF-β, and lead to cell-mediated immunity, such as macrophage activation and inflammatory responses. Furthermore, Th1 cells provide a helper function for class switch of antibody-producing plasma cells, particularly those involved in opsonization and virus neutralization [85]. In the presence of parasitic pathogens, extracellular bacteria and allergens, naïve T cells are differentiated into Th2 cells. Th2 type cytokines, including IL-4, IL-5, IL-10 and IL-13, mediate humoral immunity and support the production of the IgG1 and IgE subclasses. The Th2 cells are the cells that can interact with B cells. Upon interaction, the T cells start producing CD40 ligand (CD40L) which can interact with CD40 on DCs and B cells. In this way, the activation of more T cells and the production of antibodies are sustained [86].

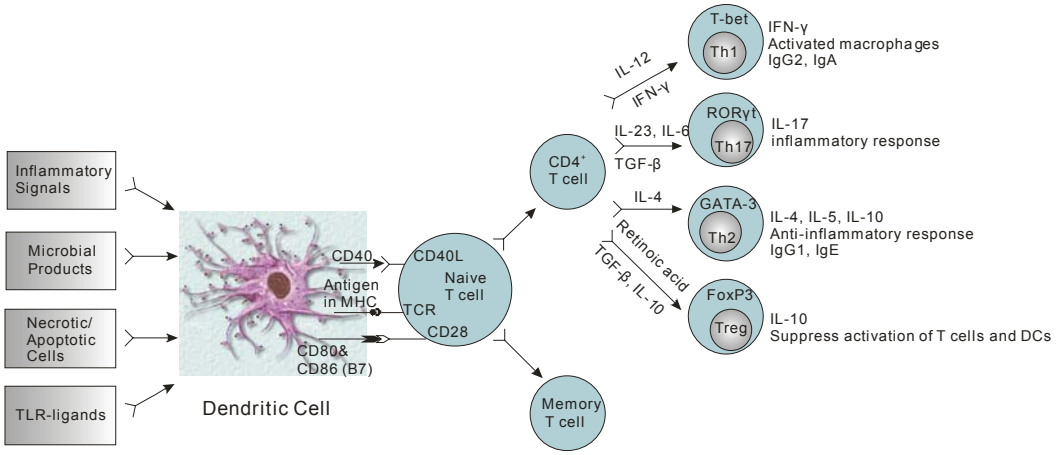

Figure 3. The adaptive immunity controlled by DCs. The specific pathway followed by $CD4^+$ T cells, whether it involves Th1, Th2, Th17 or Treg cell differentiation, is significantly governed by DCs and depending on the nature of the invading pathogens.

Th1 and Th2 cells are reciprocally regulated by a range of cytokines produced by themselves or by cells of the innate immune system. With the discovery of Th17 cells and the increasing role of antigen-induced Treg cells in controlling diseases [87], the relative simplicity of the Th1/Th2 paradigm needs modification. Nevertheless it still provides a model and reference for understanding disease pathogenesis and host immunity. The dominant type of immune response induced is determined by many factors, including the

route of antigen delivery, antigen doses, duration of antigen presentation, number, or frequency of immunizations and inclusion of adjuvants.

3.4.3. $CD8^+$ T cells

Naïve $CD8^+$ T cells become cytotoxic T cells when they are activated by DCs presenting antigens in the context of MHC I in the lymph nodes. Upon activation they migrate back to the sites of infection, where their main function is to kill tumor cells or cells infected by viruses or intracellular bacteria. The activation of a cytotoxic T cell response is the main mechanism of vaccines developed for cancer therapy. $CD4^+$ T cells seem to be required to help $CD8^+$ T cells fight certain pathogens. Cross-talk between both types of T cells is also mediated by CD40-CD40L interactions [88].

3.4.4. Memory cells

The basis of vaccination lays in the existence of memory B and T cells. These cells enable faster and stronger responses to pathogen-derived antigens encountered before [89]. These cells are long-lived and almost do not divide. However, upon contact with a familiar antigen, they start dividing quickly and induce secretion of large amounts of antibodies and/or cellular responses. This process is nicely illustrated by the enhanced immune response obtained after booster vaccinations. More recent knowledge on memory cells can be found in a recent review by Sallusto and Lanzavecchia [90].

3.4.5. Skin DCs in adaptive immunity

Under inflammatory conditions, LCs and/or the langerin positive $CD103^+$ dDCs are highly efficient at inducing cytotoxic high-avidity $CD8^+$ T cells [37, 91]. LCs are strong activators of naïve $CD4^+$ T cells, inducing their polarization into Th1 or Th2 cells. However, they are not able to promote the development of naïve B cells into IgM-secreting plasma cells [91]. In contrast, dDCs induce the differentiation of naïve B cells into IgM-secreting plasma cells through the secretion of IL-6 and IL-12, but are not very efficient at priming naïve $CD8^+$ T cells [91, 92]. Dermal DCs preferentially activate $CD4^+$ T cells, which help immunoglobulin production by B cells. LCs and dDCs appear to be equally potent at activating the proliferation and differentiation of memory T and B cells. More specifically, it is demonstrated that dDCs migrate into the outer paracortex of the lymph nodes, just beneath the B cell follicles, whereas LCs

migrate into the T cell-rich inner paracortex [93, 94]. Therefore, in summary, dDCs preferentially induce humoral immunity, while LCs and $CD103^+$ dDCs induce cellular immunity. This concept is of particular importance in vaccine formulation design and delivery for selective activation of the desired type of immune response.

Besides being presented by migratory skin DCs, soluble antigen, however, can directly diffuse into the draining lymph nodes through lymphatics and reach the lymph node-resident DCs [93]. Murine studies suggest that these two waves of antigen delivery to lymph nodes yield different immune responses. DCs can also activate innate immune cells such as natural killer cells [95, 96] and natural killer T cells [97].

4. Transcutaneous immunization

To be efficient, TCI faces at least two main challenges: the transport of antigen and adjuvant across the skin barrier, and subsequently the stimulation of the antigen uptake by DCs, as well as DC maturation and migration in an appropriate manner. Efforts are classified into two categories: i) physical/chemical methods to overcome the skin barrier; ii) co-administration of adjuvants to potentiate and redirect the immune response.

4.1. Overcoming the skin barrier

Disruption of the skin barrier increases the transcutaneous permeation of antigen and makes it more readily available for sampling by APCs. Furthermore, disruption of the skin barrier beyond a certain extent may be considered as physical trauma by the immune defense system of the skin. Danger signals, such as HSPs or the hyaluronic acid fragment (Table II), induce the secretion of pro-inflammatory cytokines by the keratinocytes and facilitate APC activation, resulting in improved immunogenicity of topically applied vaccines [98, 99]. The physiological differences between mouse and human skin should be taken into consideration when transferring techniques of skin barrier disruption from one to the other. Between the species, the density of the characterized LCs resident in the skin is comparable [100, 101]. However, human skin is thicker and less hairy than mouse skin [100, 102]. Correspondingly, the depth of LC locations is greater in humans. The physical and chemical approaches utilized to overcome the skin barrier and improve the effect of immunization are discussed below (Fig. 4).

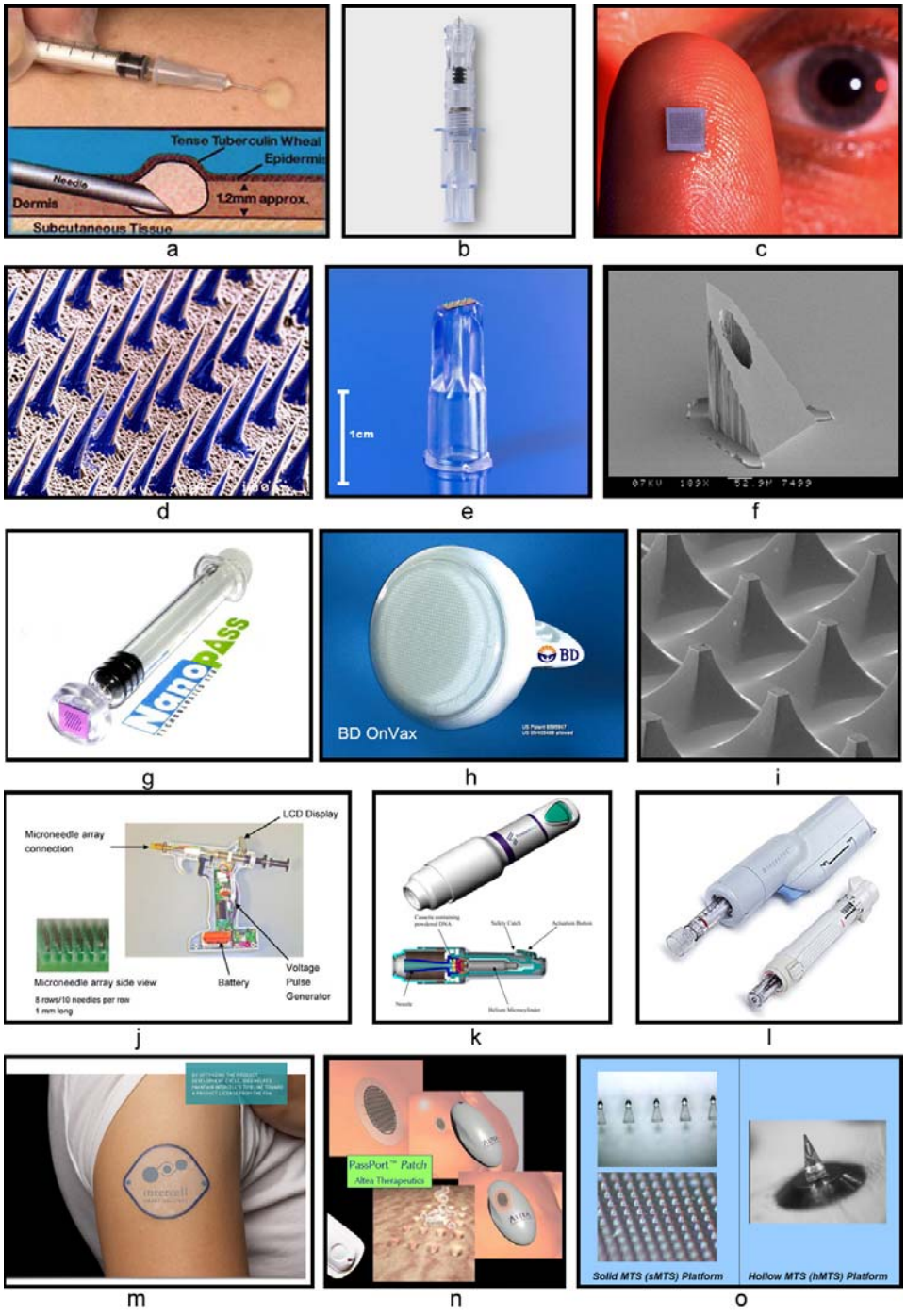

Figure 4. Approaches and devices for TCI. (a) i.d. immunization; (b) Soluvia™ (BD) [103]; (c & d) Micro-Trans™ , solid microneedle array and its SEM image [104]; (e, f & g) hollow microneedle array, MicroJet® (NanoPass) [105, 106]; (h & i) blunt-tipped microneedle array, OnVax® (BD) and its EM image [107]; (j) microneedle array with electroporation, EasyVax® [108]; (k & l) powder and liquid jet systems [109, 110]; (m) smart vaccine patch from Intercell [111]; (n) Passport™ patch (Altea) [110]; (o) coated and hollow microneedle arrays (3M) [112].

4.1.1. Intradermal injection

The most widely used method to overcome the skin barrier for cutaneous immunization to date is intradermal (i.d.) injection, invented by Mendel and Mantoux in the early 1900s [113] (Fig. 4a). It is able to deliver antigens into the dermis precisely and reproducibly. Clinical trials with hepatitis B, influenza, and therapeutic cancer vaccines have shown that i.d. vaccination is safe and effective. In many cases, benefits such as stronger immune responses with a lower antigen dose compared to subcutaneous (s.c.) or i.m. injection were observed. These underline the effectiveness of the skin as a site of immunization [3]. However, traditional i.d. injection requires well-trained, skillful healthcare workers; therefore new devices for i.d. injection are being developed. One example is the BD (Becton Dickinson) microinjection system, Soluvia™ (Fig. 4b). This is a prefilled syringe with a single 1.5 mm-long, 30G intradermal needle designed to deliver 100-200 µl fluid. It is now commercially available for a trivalent seasonal influenza vaccine (Sanofi-Pasteur) [114]. However, it still employs needles and causes pain. Cutaneous immunization in a minimal-invasive and needle-free manner is therefore more desirable.

4.1.2. Microneedle arrays

One approach towards painless and needle-free TCI is to dramatically reduce the size of needles so that they are barely perceptible. The term microneedles in the definition used here refers to needles shorter than 1 mm with a cross-sectional diameter of about 300 µm or less. Theoretically, microneedles only need to pierce the 15-20 µm thick *stratum corneum* before reaching the viable epidermis. However, the skin is elastic, heterogeneous tissue and slightly stretched *in vivo.* The mechanical and structural properties of the skin vary significantly with age, skin type, hydration level, body location and among individuals [115, 116]. To ensure effective and reproducible piercing regardless of these factors, microneedles need to be fabricated much longer than 20 µm [117]. The diameter of the microneedle is also important. A too small diameter can only provide limited diffusion flux. Moreover, very thin microneedles are normally very fragile and may easily break in the skin. To overcome this risk, microneedle arrays were designed, which can help to spread the surface forces between each microneedle, thereby decreasing the chances of breakage in the skin. More importantly, by using an array of

microneedles, more conduits are created in the skin, thereby increasing transcutaneous diffusion of antigen and exposing more APCs.

The concept of the microneedle array for drug delivery purposes essentially dates back to a patent, filed in 1971, by Gerstel and Place at Alza Corp [118]. However, it was not until the 1990s that the technique became viable, as by then techniques became available to precisely fabricate these microneedle arrays in a potentially cost-effective manner. Since then, microneedle technology is under active research and various strategies were developed using microneedle arrays in transdermal drug delivery, including TCI [119, 120].

Solid microneedle arrays

A straightforward method is to perforate the skin with solid microneedle arrays and apply antigens to the skin surface for subsequent diffusion into the skin. Henry *et al.* demonstrated four orders of magnitude increase in permeability for calcein and bovine serum albumin (BSA) through human epidermis *in vitro* after penetration with a microneedle array of 150-μm needle length [121]. Banks *et al.* reported that the flux across microneedle array-pretreated skin was augmented by increasing the charge of the drug [122] and Verbaan *et al.* showed that 200-nm particles can diffuse through conduits formed by solid microneedle arrays [123].

Coated microneedle arrays

Besides pretreatment, arrays of microneedles with vaccines coated in the form of powder or a film have been developed. Although only a very low amount of antigen can be coated, this may be sufficient to generate a protective immune response. The coated microneedle array is inserted into the skin and then removed, thus depositing its payload to a maximum depth determined by the length of the microneedle. Matriano *et al*. delivered 1 μg ovalbumin (OVA) by precoated microneedle arrays and showed up to a 100-fold increase in immune responses over i.m. injection of the same dose [124]. In that study, an array with 300-μm long microneedles, made of titanium, was applied to the skin by an impact insertion applicator. Later, Widera *et al.* from the same group carried out an extensive study on microneedle fabrication parameters. The immune response was found to be dose dependent, however, practically independent of depth of delivery, density of microneedles, or area of application. Notably, OVA delivered with short

microneedles (225 μm) in a high density array (725 microneedles/cm^2) induced a similar immune response as compared to longer microneedles (600 μm) at a lower density (140 microneedles/cm^2) [117].

Coatings are usually applied by dipping microneedles in the vaccine formulations. A systematic study performed by Gill and Prausnitz demonstrated that excipients reducing surface tension of the coating solution improve coating uniformity, while excipients increasing solution viscosity increase coating thickness. The amount of coated antigen can be adjusted by its concentration in solution. Both hydrophilic and hydrophobic molecules could be uniformly coated onto microneedles. Coatings could be localized just to the needle shafts and formulated to dissolve within 20 s in porcine cadaver skin [125, 126]. More recently, Chen and his coworkers reported a novel gas-jet coating method, with which they achieved uniform coating of a wide variety of molecules, *e.g.* ethidium bromide (394 Da), OVA (44 kDa) and OVA-encoding DNA (3.2 MDa), to microneedle arrays (30 to 90 μm needle length) [127]. As they used arrays of very small and densely packed microneedles, they claimed that only the gas-jet coating method, but not the dip-coating method, was able to localize the coating primarily to the needle shafts instead of the back plate. In the same study, they performed TCI with OVA on mouse ear skin using two dip-coated microneedle array-containing patches (3364 needles/16 mm^2, delivering 1.2 μg OVA each) and induced comparable antibody titers to those from i.m. injection of 6 μg OVA.

Hollow microneedle arrays

By solid microneedle arrays pretreatment, antigen delivery is based on passive diffusion along the conduits. Although this is a relatively easy approach from a technical point of view, in general it leads to a low bio-availability of the applied vaccines. Using hollow microneedle arrays to inject the vaccine into the epidermis or the superficial layers of the dermis, one can precisely steer the flow rate using a syringe or a pump and provide a more controlled vaccine delivery. However, avoiding leakage is the biggest challenge for a hollow microneedle array due to the short needle length. A hollow needle with the opening facing the skin will punch out a piece of tissue. This leads to blockage of the fluid path. A large fluidic pressure applied by a piston or pump against this resistance will cause leakage. By geometrically shaping the needle tip and partially retracting the needles after insertion, thus avoiding blockage and relieving the compressed tissue, the flow resistance

can be decreased substantially [128]. The first hollow microneedle array, 150-μm long, made of silicon, was presented by McAllister *et al.* [129]. Luttge *et al.* reported injection of insulin in diabetic rats through a 350-μm long, 9×9, silicone microneedle array *via* a pump at a rate of 0.045 mg/h (estimated to be >1 μl/h based on the solubility of insulin [130]). Comparable reduction of glucose levels were achieved as compared to conventional s.c. injection (Fig. 4f) [105]. Hafeli *et al.* demonstrated injection of radio-labeled human serum albumin (HSA) into mouse skin using 200-μm long, 2×3 microneedle arrays. After injection of about 3 μl fluid, the resulting relative skin uptake (the volume in the skin divided by the ejected volume) was 36.0 ± 19.9% [131]. Lower doses (3 μg) of influenza vaccines delivered by a hollow microneedle array (0.45-μm long, 4×1, Microjet® developed by Nanopass, Fig. 4e) elicited immune responses similar to those induced by full-dose (15 μg) i.m. vaccination in human volunteers [106]. In this study, a blanched bleb appeared after injection. Leakage was noted during injection in 7 out of 60 subjects, without significantly affecting the immune response.

Dissolvable microneedle arrays

Another design is the dissolvable microneedle array. Kolli *et al.* tested 500-μm long microneedles made of maltose. They demonstrated that microchannels in the skin were created and about a ten-fold increase of the transdermal delivery of nicardipine hydrochloride was achieved [132]. The VaxMat®, made of sugar matrix containing vaccines by TheraJect Inc., are fabricated in various lengths from 100 μm to 1,000 μm and assembled with an adhesive patch. Upon piercing, the microneedles dissolve and antigen diffuses into the epidermis and dermis within minutes [133].

Combined approaches using microneedle arrays

The BD's OnVax® device employs blunt-tipped microneedles measuring 50–200 μm in length over a 1 cm^2 area (Fig. 4h and 4i). These "microenhancer arrays" were coated with vaccines and used to scrape the skin gently in order to expose LCs to the vaccine without pain sensation. Using a hepatitis B DNA vaccine-coated microneedle array (100 μg dose), stronger and less variable immune responses were achieved compared to conventional i.m. and i.d. injection. Moreover, 100% of seroconversion was achieved after only two immunizations, whereas only 40-50% conversion was obtained by the conventional techniques, unless more immunizations were

applied. This enables “wipe and go” vaccination with easy self-administration [107].

The EasyVax™ device has been designed to insert coated microneedle arrays into the skin followed by electrical pulses to deliver DNA into the cells (Fig. 4j). Mice vaccinated with smallpox DNA vaccine induced neutralizing antibody titers greater than those elicited by the traditional live virus vaccine administered by scarification [108].

Some trends can be noticed after ten years study in this field:

i) instead of piercing on dermatomed skin *in vitro*, recently more relevant and adequate experimental evaluations are being performed *in vivo;*

ii) the length of the microneedle falls more often in the range of 200-500 μm, which allows effective piercing with less pain sensation;

iii) an impact applicator or insertion device is often used, which enhances the uniformity of skin piercing with shorter needle length;

iv) hollow microneedle arrays have gained more attention for its potential of precise dose control, while the device needs to be improved with respects to leakage-free injection and simplicity.

4.1.3. Tape-stripping

Tape-stripping and skin abrasion employs adhesive tape or emery paper to (partially) remove the *stratum corneum*. Glenn *et al.* have reported that mild abrasion by making 15 strokes on the skin surface results in the removal of approximately 29% of the *stratum corneum*, which can greatly enhance the passive diffusion of an antigen. This study confirmed that *stratum corneum* disruption before applying a vaccine patch (containing 50 μg heat-labile enterotoxin from *E. coli*, LT) results in robust immunity comparable to that obtained after active toxin infection and immunity induced by oral cholera vaccine [134]. For reproducible and easy-to-use tape-stripping, a skin preparation system (SPS) has been developed by Iomai (current Intercell). In the SPS device, an abrasive strip of fine-grit sandpaper is used to provide mild and controlled *stratum corneum* disruption by the length of the strip with only one stroke instead of 15 strokes. Following the same immunization protocol, comparable LT-specific antibody titers were obtained from the groups treated by trained physicians and a self-treated group [135]. Cyanoacrylate skin surface stripping (CSSS) facilitates more the follicular

penetration by removing cellular debris and sebum from the hair follicle openings, thereby enhancing vaccines to be delivered to the follicular LCs [136]. It is reported that topical application of modified vaccinia Ankara particles (~290 nm) after CSSS pretreatment induced protection against vaccinia virus challenge in mice [137].

4.1.4. Jet injection

Powder jet injection employs the PMED™ device to deliver vaccines (Fig. 4k, Pfizer), formulated as dry powder, mainly to the epidermis by releasing compressed helium at 40 bar pressure from a gas cylinder. This route of vaccination is referred to as epidermal powder immunization (EPI) in the following discussion. Liquid jet injection uses liquid vaccine formulations instead of the powder to puncture the skin and deliver vaccines without the use of needles (Fig. 4l). This technique was invented in the 1860s and the multi-use-nozzle jet injection was introduced in 1950s, developed by the U.S. military. Billions of vaccines doses have been administered by this method until in 1985, when it was related to a large hepatitis B outbreak [138]. This abandoned technique now resurrects with safer design, *e.g.* disposable cartridges prefilled with vaccines [139].

4.1.5. Ultrasound

Tezel *et al.* applied low-frequency ultrasound to disrupt the skin barrier till the skin resistance decreased from 60 to below 5 kΩ/cm^2 [140]. Functioning as a physical adjuvant, ultrasound enhanced the immune response induced by topical application of 100 μg TT in mice, probably by enhancing the antigen transport across the skin barrier and the activation of LCs. It generates a potent systemic immune response without using a toxin adjuvant or skin abrasion.

4.1.6. Electroporation

Electroporation of intact skin involves transmitting high-voltage electrical pulses to disrupt lipid structures, thereby creating transient pores in the lipid regions of the *stratum corneum*. It has been reported to enhance the permeation of highly-charged macromolecules (heparin) across the *stratum corneum* reaching therapeutic levels [141]. Electroporation was found to stimulate the exodus of LCs from the skin, which may be an additional

advantage for vaccination purposes. TCI of 130 µg OVA-peptide with 100 µg CpG oligo deoxynucleotides (CpG) as an adjuvant by electroporation into mouse skin was shown to generate a strong cytotoxic T-cell response comparable to that induced by i.d. injection of the antigen with Freund's complete adjuvant [142]. Electroporation also permeabilizes the viable cells, thereby increasing the uptake of the antigen. However, with the formation of transient pores in the *stratum corneum* during electroporation, resistance can drop rapidly and dramatically. Therefore the electric field may distribute to the deeper tissues, causing pain and muscle contractions, especially at higher pulse voltages required for pore formation. Although this can partially be avoided by using closely spaced microelectrodes to constrain the electric field within the *stratum corneum*, the use of electroporation in TCI for human is limited by the complexity of device design [143].

4.1.7. Thermo-ablation

Thermo-ablation makes tiny conduits by burning away small micrometer-sized areas of the *stratum corneum*. This can be obtained by pulsed laser [144], arc discharge [145] or short-duration resistive heating [146]. The latter is employed by the PassPort^{TM} system, commercialized by Altea Therapeutics Corp (Fig. 4n). The use of this system creates 80 micropores within a 1-cm^2 area with a filament attached to an applicator for the electrical current supply. This area is covered with a disposable liquid reservoir patch containing vaccine formulation. TCI using this system by application of 3 µg of recombinant H5 influenza hemagglutinin and 25 µg CpG three times with 4 week intervals induced robust serum antibody responses in mice and provided protection against a lethal challenge with a highly pathogenic avian H5N1 influenza virus [146].

4.1.8. Chemical approaches

Water is one of the most frequently used penetration enhancers. Occlusion and hydration of skin tissue progressively increases its permeability, as hydrated *stratum corneum* results in swelling of the corneocytes, pooling of fluid in the intercellular spaces and dramatic microscopic changes in its structure at very high hydration levels [147]. Consequently, methods such as occlusive patches or hydrophobic ointments (*e.g.* vaseline) also lead to

increased skin permeability. Occlusive patches have been successfully utilized and combined with various TCI approaches [148-150] (Fig. 4m).

Other penetration enhancers act by diminishing the barrier of the skin. A great variety of chemicals are known to posses this capability as reviewed by Williams and Barry [151]. More recently, six hundred formulations of commonly used chemicals were screened for their potency in both transcutaneous permeation enhancement and adjuvanticity by Karande and his coworkers. Methodology described in this study provides a rational strategy for the design of TCI formulations by testing chemicals on both permeation-enhancing properties and adjuvanticity *in vitro.* OVA formulated with chemical or mixture of chemicals superior in both properties showed higher immunogenicity *in vivo*. Notably, chemicals with either high permeation-enhancement potency or high adjuvanticity alone did not guarantee high immunogenicity [152].

4.1.9. Deformable vesicular antigen delivery systems

The vesicular antigen delivery systems, as a combined physical/chemical approach, have also been exploited to enhance the permeation of antigens in TCI. These vesicles are reported to pass through pores/tunnels smaller than their actual size, owing to their highly deformable bilayer [153]. They also have the potential advantages of boosting the immune response because of their similar size and structure to microorganisms, the natural pathogens which are actively sampled by the APCs [154].

Transfersomes®

Transfersomes® are ultradeformable liposomes. Liposomes are closed spherical structures consisting of bilayers of hydrated amphiphilic lipids. Liposomes have first been identified as adjuvants in 1974 [155]. They may exert their adjuvanticity by providing a sustained antigen release, epitope multimerization and particulate antigen delivery to APCs. Liposomes, especially cationic liposomes, have been extensively explored as carriers for protein and DNA vaccines as they can carry both membrane-associated and water soluble antigens [156, 157].

The ultra deformability is generated by incorporation of an edge activator, often a surfactant, in the lipid bilayer [158, 159]. The original composition of Transfersomes® was soybean phosphatidyl choline (SPC) with sodium

cholate and small amount of ethanol [160]. Transfersomes® are applied in a non-occlusive manner as it has been suggested that the hydration gradient in the s*tratum corneum* will drive the intact vesicles into the viable epidermis [161]. However, the claim has not yet been substantiated [153]. Structural changes in the *stratum corneum* have been identified and vesicle structures have been visualized within the *stratum corneum* lipid regions, but no intact vesicles have been ascertained in the viable tissues [153]. Nevertheless, several groups have reported that Transfersomes® substantially increase the transport of small molecules across the *stratum corneum* [158, 162-164].

The use of Transfersomes® to formulate antigens in TCI has also been reported in a few studies. When using antigens such as HSA, gap junction protein (GJP) and TT, potent humoral immune responses were induced in murine models with antibody levels comparable to those obtained through s.c. injection of HSA, GJP in Transfersomes® and alum-adsorbed TT, respectively [165-167]. Transfersomes® (named elastic liposomes by Mishra *et al.*), prepared with SPC, Span 80 and ethanol, were loaded with hepatitis B surface antigen (HBsAg). Comparable IgG titers and much higher secretory IgA titers against HBsAg were induced when elastic liposomes loaded with 10 µg HBsAg were applied onto intact mouse skin as compared to those obtained by i.m. injection of the same dose of alum-adsorbed HBsAg [168].

Other elastic vesicles

A number of other types of elastic vesicle compositions have also been evaluated in TCI, *e.g.* with high percentage of ethanol being introduced into the vesicles, the ethosomes; or constructed from non-ionic surfactant and cholesterol, the niosomes. Ethosomal systems were shown to be much more efficient at delivering a fluorescent probe to the skin in terms of quantity and depth, than either conventional liposomes or a water/ethanol solution. TCI of HBsAg-loaded ethosomes has been reported to induce immune response comparable to i.m. injection of HBsAg-alum [169]. BSA-loaded niosomes, composed of sorbitan monostearate/sorbitan trioleate (Span 60/Span 85), cholesterol and stearylamine, were coated with a modified polysaccharide O-palmitoyl mannan (OPM) for targeted delivery to the LCs. This niosomal formulation elicited significantly higher serum IgG titers as compared with alum-adsorbed BSA and plain uncoated niosomes in TCI, but lower than those obtained after i.m. injection of BSA-alum [170].

Van den Bergh *et al*. introduced a series of surfactant-based elastic vesicles, consisting of a bilayer-forming surfactant sucrose-laurate ester (L-595), an edge activator octaoxyethylene-laurate ester (PEG-8-L) and a charge inducer sodium bistridecyl sulfo succinate (TR-70) [171, 172]. It has been suggested that these elastic vesicles act as carrier systems to transport low-molecular-weight drugs into the *stratum corneum* [173-176]. Studies using freeze fracture electron microscopy have visualized channel-like regions together with vesicular structures in the deep layer of *stratum corneum* after non-occlusive treatment with elastic vesicles [172]. Therefore, there is a potential for antigen-loaded vesicles as effective formulations for TCI, although there is no evidence that vesicles diffuse intact into the viable epidermis.

Table IV. New technologies targeting vaccine delivery into the skin

a. Microneedle related approaches

Technology	Vaccine (development phase)	Company or Ref
Soluvia™, (prefilled microinjection)	Trivalent inactivated seasonal influenza vaccine (clinical phase III)	BD/Sanofi-Pasteur
	Cancer vaccine (clinical phase II)	BD/Oncovax
Microneedle injection	Anthrax vaccine (pre-clinical)	BD, [177]
Micro-Trans™, (solid microneedle array)	DNA, OVA, influenza (pre-clinical)	BD, 3M, Valeritas/biovalve, [121]
OnVax® (coated, blunt-tipped microneedle array)	Hepatitis B DNA vaccine (pre-clinical)	BD, [107]
Macroflux® (coated microneedle arrays)	OVA (pre-clinical)	Alza, 3M, Zosano, [124], [117]
MicronJet® (hollow microneedle array)	Influenza (clinical phase I)	Debiotech, 3M, NanoPass, [106]
EasyVax® (microneedle array with electro-poration)	Smallpox DNA vaccine (pre-clinical)	[108]
VaxMat® (dissolvable microneedle vaccine array)	Not available	Theraject, [133]

b. Other physical and chemical approaches

Technology	Vaccine/(development phase)	Company or Ref
SPS (topical patch and skin abrasion)	Trivalent inactivated seasonal influenza (clinical phase II)	Iomai/Intercell
	Heat-labile enterotoxin from *E. coli* (LT) for travelers' diarrhea (clinical phase III)	[135, 178]
	Influenza (clinical phase II)	[179]
	Anthrax (pre-clinical)	[149]
	DT (pre-clinical)	[180]
CSSS	Melanoma or HIV epitopes (clinical phase I)	[181]
Electroporation	OVA peptide (pre-clinical)	[142]
Inovion, MedPulser DNA delivery system	DNA dengue (clinical phase I)	
PassPort™ system (topical patch with thermo-ablation)	Influenza & DNA (pre-clinical)	Altea, [146]
PMED™ (powder jet injection)	DNA HIV (preclincal)	[182]
	Herpes simplex virus (HSV) type 2 (clinical phase I)	[183]
	DNA melanoma gp100 (clinical phase I)	[184]
	Influenza DNA vaccine (clinical phase I)	[185]
	Hepatitis B DNA coated gold micro-particles (clinical phase II)	[186]
	Influenza (clinical phase I)	[187]
Biojector® 2000 (liquid jet injection)	Inactivated polio vaccine (IPV) (clinical phase II)	Bioject, [188]
	DNA vaccines for cancer, HIV &	[189]
	Protein (clinical phase I)	[190]
Low frequency (20KHz) ultrasound	TT (pre-clinical)	Sonics & Materials, [140]

The stages of development of the approaches mentioned are summarized in Table IV. The long list of strategies/devices developed to overcome the skin barrier and enable painless, needle-free TCI reflects a very competitive and fast developing field.

4.2. Immune potentiators and modulators

Adjuvants, immune potentiators and modulators, are substances that enhance the immunogenicity of an antigen. Due to the advances in understanding innate immunity, the range of adjuvant candidates is enlarging dramatically. In many established, as well as experimental vaccine formulations, ligands for PRRs, cytokines or messenger molecules involved in the signal transduction of PRRs are incorporated, as reviewed by Wilson *et al.* [10]. As the route of administration determines the targeted subgroup of APCs, the immune modulation effectuated by adjuvants may differ depending on the site of vaccination. For example, in general, mucosal administration of antigen and adjuvants induces secretory-IgA which provides mucosal protection. Some representative adjuvants and the biased antibody isotypes in lab animals are listed in Table V, corresponding to the route of administration and their basic mechanisms of action. Their immune modulation properties in TCI on microneedle-treated skin will be further studied in this thesis.

4.2.1. Bacterial exotoxins

Bacterial ADP-ribosylating exotoxins possess a high degree of immunogenicity and adjuvanticity. Among them, cholera toxin and LT are the ones most intensively studied [190].

CT is a protein molecule consisting of five nontoxic B subunits (CTB) surrounding a single, toxic A subunit (CTA). Both the CTB-mediated specific binding to the GM1-ganglioside receptor and the ADP-ribosyl transferase activity of CTA were reported to be of importance in the immune stimulatory properties of CT [191, 192]. CT is a predominantly Th2 biased immune modulator when co-administrated with antigens by the intravenous route, oral administration or co-cultured with human blood monocytes-derived DCs *in vitro* [192]. Although diarrhea associated with CTA has prevented its use as a mucosal adjuvant, topical application of a high dose of CT does not appear to result in toxic side effects [193].

Table V. Antibody isotype bias induced in lab animals (mainly rodents) of selected adjuvants, the corresponding route of administration, and their basic mechanisms of action.

Adjuvants	Basic characteristics and target	Delivery route	Dominant isotype	Ref
$Al(OH)_3$, $AlPO_4$	Nalp3 inflammasome	i.p.*	IgG1 & IgE	[23] [24]
	and uric acid,	s.c.	IgG1	[194]
	depot effect	EPI	IgG1	[195]
QS21/QuilA	Purified saponin	i.m.	IgG2a & IgE	[196]
		Intranasal	IgG2a & IgA	[196]
		s.c.	IgG2a	[194]
		Oral	IgG1, IgG2a, & IgE	[197]
Immune stimulatory complex (ISCOM)	40 nm cage-like particles, depot effect	Parenteral	IgG2a	[198]
		Intranasal	IgG1, IgG2a & IgA	[199, 200]
Monophosphoryl lipid A (MPL) & LPS analogs	TLR4	Intranasal	IgG2a & IgA	[201]
		s.c.	IgG2a	[202]
Cholera toxin & B subunit of CT (CTB)	GM1-ganglioside	i.m.	IgG1	[203]
		TCI	IgG1 & IgG2a	[204]
		Oral	IgG1 & IgA	[205]
		i.v.**	IgG1	[205]
CpG	TLR9	i.m.	IgG2a	[206]
		s.c.	IgG2a	[207]
		TCI	IgG2a	[208]
		Intranasal	IgG2a	[209]
		EPI	IgG2a	[195]

*i.p.: Intraperitoneal injection
**i.v.: intravenous

LT shares 82% of amino acid homology with CT and both bind to the GM1-ganglioside receptors preferentially on the DCs *in vivo* [201, 210, 211]. LT induces a stronger Th1 response than CT [210]. An immune stimulatory patch containing LT has been shown to enhance the immune protection induced by i.m. injection of influenza vaccine in the elderly [179]. The clinical phase I trial of an LT patch has been shown to be effective in ameliorating the symptoms of traveler's diarrhea [212].

4.2.2. CpG

Prokaryotic DNA contains unmethylated CpG dinucleotides within nucleic acid motifs that are recognized by the innate immune system of vertebrates [213]. These immune stimulatory motifs are the ligands for TLR9, found primarily in intracellular vesicles of phagocytic cells [213]. By signaling through TLR9, CpG induces production of reactive oxygen species and activation of NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells), followed by the secretion of pro-inflammatory cytokines such as IL-12, TNF-α and IFN-γ, resulting in a Th1 biased response [207, 214]. CpG motifs are capable of stimulating secretion of immunoglobulins and modulating pre-existing immune responses [207, 215]. Therefore, synthetic CpG has been considered as candidate immune modulatory adjuvant. CpG has been included in many experimental vaccines and demonstrated enhanced protection against a variety of pathogens including Ebola virus, *Bacillus anthracis*, *Francisella tularensis, L. monocytogenes*, *Cryptococcus neoformans*, malarial antigens, anti-*H. influenzae* glycoconjugates and melanoma antigens [207, 216-220]. CpG has shown adjuvant activity in combination with dermally and mucosally delivered antigens as well [208, 209].

4.2.3. LPS

LPS, the major outer membrane constituent of Gram-negative bacteria, stimulates APCs through TLR4 [221]. LPS induces high level production of pro-inflammatory cytokines. A drawback of LPS is its toxicity and pyrogenicity in humans. Therefore, detoxified forms of LPS were developed, such as monophosphoryl lipid A (MPL), developed by removing a phosphate group, sugar moiety and an ester-linked fatty acid group; and *lpxL1* LPS, containing penta- instead of hexa-acylated lipid A. These LPS derivates show less toxicity, while retaining their immune stimulatory properties [222, 223]. Similar to LPS, MPL interacts with TLR4 on APCs and induces strong, mixed Th1/Th2 responses with a bias to Th1, and cytotoxic T cell responses [224]. Since freshly isolated human LCs do not express detectable levels of TLR4 and do not mature in response to its LPS ligands, LPS and its detoxified analogs are of research interest for studying antigen delivery and immunological mechanisms in TCI.

4.2.4. Virus-like particles

Virus-like particles (VLPs) are viral proteins, such as capsid proteins, that spontaneously form particles resembling virions. They are relatively stable and inert particles. They do not contain encapsulated viral genes that could be potentially harmful. However, the constituent viral capsid proteins retain their native conformation and receptor-binding capacity and are therefore highly immunogenic [225]. Antigens delivered by VLPs have the potential to be presented *via* MHC II following endosomal processing, but endosomal escape into the cytosol will also allow for antigen presentation *via* the MHC I pathway [225]. This allows for induction of both humoral and cell-mediated immunity. Young *et al.* immunized mice on intact skin with VLPs in combination with CT and CpG. Antigen-specific IFN-γ secretion and secretory-IgA on the mucosal surface were induced as well as systemic IgG1 [226].

4.2.5. Quil A

Quil A, a saponin-based adjuvant, is composed of immune stimulatory fractions extracted from the bark of the tree *Quillaja saponaria.* QS21 is a purified saponin fraction from Quil A [194]. Besides their immune stimulatory properties, saponins interact with lipids of cell membranes and cause cell lysis. The tissue-reactive toxic nature has plagued their development as adjuvants [227]. By mixing phospholipids and cholesterol with saponins under controlled conditions, 40 nm cage-like particles, referred to as ISCOMs, can be created. They were first described in 1984 by Morein *et al*. [198]. ISCOMs have been shown to promote both humoral and cellular immune responses with several different antigens. As TCI does not introduce direct contact between vaccine and adjuvants with the general blood circulation, Quil A, QS21 and ISCOMs might be safely combined with TCI for further formulation development.

4.3. Combined approaches for improving TCI

Approaches discussed so far can be combined for the sake of improving TCI and tuning the immune responses for specific preventive or therapeutic needs. Synergy between different families of immune potentiators and modulators has long been studied and widely used in vaccination practice. In AS04, an adjuvant system developed by GlaxoSmithKline, MPL was added and adsorbed onto aluminum hydroxide or aluminum phosphate, thereby skewing

the Th2 biased response induced by the alum towards the Th1 direction. It is now marketed in vaccines against viral infections, such as FENDrix™ (hepatitis B) and Cervarix™ (human papillomavirus, HPV) [228]. Immune modulators may also be combined with antigen delivery systems. Schlosser *et al.* reported that co-encapsulation of CpG or PolyI:C with OVA into the same PLGA particles induced higher antigen-specific, cytotoxic T-cell responses than its addition in a soluble form, most likely by targeting antigen and adjuvant to the endosomes within the same cell [229]. In AS01, another adjuvant system from GlaxoSmithKline, MPL and QS21 was incorporated into liposomes with the aim to favor Th1 responses and improve the $CD8^+$ cell mediated immunity. The AS01 formulation of RTS,S (recombinant fusion of circumsporozoite protein and HBsAg) is now being evaluated in a phase III field study against malaria [230, 231]. Antigen-adjuvant conjugates and antigen-adjuvant fusion constructs are also potential candidate formulations for TCI [232, 233]. With the advance in understanding the functional specialization of skin DC subsets, immune modulation by targeted delivery of antigen and adjuvant predominantly to one of these skin DC subsets is theoretically possible yet challenging. For instance, tape-stripping and microneedle arrays with very short needle lengths will expose mainly LCs to the antigens following TCI, whereas ligands binding to specific receptors may be utilized to home antigen to a single skin DC subset.

4.4. Safety concerns

Most of the skin barrier disruption approaches mentioned are simple and hygienic in practice as most of them employ disposable devices or cartridges for single application only. Disinfecting the site of application with 70% ethanol beforehand and covering with patches afterwards can provide sufficient protection against potential infections and cross contaminations. Notably, the conduits created by microneedle arrays are reported to be open for up to 72 h under occlusive conditions and close within several hours when not occluded [234, 235]. This provides an option for a controlled antigen delivery through the conduits and prevention of a pathogenic contamination.

The newest and most promising vaccine developments employ a variety of strategies for immune stimulation that enhance the responses to specific antigens. However, the advantages of immune stimulation are inevitably accompanied by acute safety risks associated with systemic adverse reactions. A balance between potency and adverse reactions will need to be

achieved for widespread acceptance of human vaccines [215, 222]. The risk of different types of side reactions associated with vaccination depends on the level and Th1/Th2 balance of the immune response, as well as the administration technique [8, 236, 237]. Th1 responses are usually linked to inflammatory cellular responses. The production of IFN-γ in particular, potentially favors type IV delayed type hypersensitive reactions (the Gell and Coombs classification), such as eczema and pruritus [238]. Th2 lymphocytes that contribute to IgE antibody responses, can favor immediate or late hypersensitivity reactions, involving mast cells and eosinophils, respectively [8].

Cutaneous immunization avoids the general blood circulation while favoring lymphatic drainage of antigens. The risk of systemic shock is likely to be lower than observed for the i.m. or s.c. routes [8]. Therefore, cutaneous immunization is attractive for its potential for safe and potent immune stimulation. However, unlike injectable vaccines which have been administrated billions of times, the thorough safety profile of cutaneous immunization, including TCI, is yet to be established.

4.5. Concluding remarks

TCI provides effective, easy-to-use, painless, and needle-free vaccination with fewer side effects and safer handling than the conventional injections. The main challenges are to ensure reliable and accurate delivery of antigens into the epidermal and/or dermal skin tissue and to formulate antigens with adjuvants and/or particulate carrier systems for selective activation of the proper PRRs existing in the skin DC subsets. Joint efforts from immunologists, vaccinologists, pharmaceutical scientists, and (fine) mechanical engineers should ensure further improvement of TCI and essentially revolutionize the current vaccination practice.

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Chapter 2

Aim of the thesis and study objectives

Aim and scope of this thesis

The aim of the work described in this thesis was to improve the immunogenicity of antigens in transcutaneous immunization (TCI) by using microneedle arrays, immune-modulators, and antigen- containing vesicle formulations. The study objectives include:

i). To investigate the use of microneedle arrays to potentiate immune responses of topically applied antigen;

ii). To combine microneedle arrays with adjuvants to modulate immune responses induced in TCI;

iii). To prepare, characterize and evaluate surfactant- and lipid-based vesicle formulations for vaccine delivery across the *stratum corneum* into the viable layers of the skin;

iv). To investigate the immunogenicity of topically applied antigen using antigen-loaded vesicular carrier systems *in vivo*;

v). To improve TCI by combining antigen-loaded vesicle formulations with microneedle array pretreatment of the skin.

Organization of the thesis and experimental approaches

Chapter 1 provides a comprehensive review of the current research status of TCI, including a brief introduction to vaccines and adjuvants, the structure and barrier functions of the skin, and the innate and adaptive immunity involved in cutaneous immune defense. The main part of this chapter summarizes the strategies and experimental approaches of enhancing transcutaneous antigen delivery and improving/optimizing the immunogenicity of vaccine formulations.

Chapter 3 describes the microneedle arrays and the impact insertion applicator developed for TCI. This system was first evaluated for effective piercing of mouse skin *in vivo*. Then, the effect of microneedle array pretreatment on TCI of diphtheria toxoid and influenza vaccine is presented. The 300 μm-long assembled microneedle array is used in the research described in ***Chapter 4*** --- a comparative study on the immune potentiation and modulation functions of various adjuvants in TCI of DT onto microneedle-pretreated skin.

In ***Chapter 5 and 6*** studies are presented on the potential of DT-containing vesicle formulations in TCI. Two types of DT-containing vesicle formulations, i.e. negatively charged surfactant vesicles and positively charged (elastic) liposomes, are prepared and characterized *in vitro*. Selected formulations are tested in TCI onto intact and microneedle array-pretreated mouse skin. These vaccinations were compared with vaccination through intradermal and subcutaneous injection. Human peripheral blood mononuclear cell-derived immature dendritic cells are used to assess the immune-stimulatory potential of these formulations.

A summary of the outcome of this work, a general discussion and perspectives are provided in ***Chapter 7***.

Chapter 3

Microneedle arrays for the transcutaneous immunization of diphtheria and influenza in BALB/c mice

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Introduction

The majority of vaccines are administered parenterally, however, multiple injections by syringes and needles require trained personnel and cause stress to children and parents in the pediatric vaccination program. In case of influenza, since the virus undergoes frequent antigenic variation, the current solution of controlling this disease by vaccination requires annual injections. Multiple injections (the pediatric vaccination program and influenza vaccination) raise the demand of a patient-friendly vaccine delivery. Transcutaneous immunization (TCI) offers an attractive route as it has the advantage of improved compliance and may lead to comparable or more potent immune responses than the conventional injection [1]. An enhanced immune response is expected because the skin is populated with a high number of immune-competent dendritic cells (DCs) in the dermis and Langerhans cells (LCs) in the epidermis [2, 3]. After activation, they migrate to the draining lymph nodes, present antigens to T cells and initiate systemic humoral and cellular immune responses [4-7].

As the natural function of the skin is to protect the body against water loss and unwanted environmental influences, it is also the major obstacle for dermal delivery of vaccines. The barrier function of the skin is located in the uppermost layer of the skin, the *stratum corneum*. It consists of corneocytes embedded in a highly organized crystalline lamellar structure of the intercellular lipid matrix. This unique arrangement of the 15-20 μm thick layer results in a practically impermeable barrier for many compounds. To overcome this barrier and achieve effective TCI, formulations with potent adjuvants, particular carrier systems, penetration enhancers as well as physical methods *e.g.* thermal ablation [8], microdermabrasion [9] electroporation [10] and cavitational ultrasound [11] have been studied in animal models and human, resulting in improved antibody titers compared to non-adjuvanted vaccine on intact skin and in some cases protective immunity was achieved. Recently, phase II trial of TCI using *E. coli* heat-labile exterotoxin applied on the skin after mild abrasion was successful in mitigating traveler's diarrhea [12]. However, low immunological efficiency, safety issue of adjuvants, poor patient compliance and complexity of application still pose severe problems hindering wide-spread introduction of TCI for mass vaccination campaigns.

A fast developing technique for TCI is to increase the skin permeability using a microneedle array. It was first proposed by Gerstel and Place in the 1970s [13]. Prausnitz resumed the study using microneedle arrays in transdermal drug delivery 10 years ago when the technology of fabrication in micron dimensions became readily available [14]. Currently, it is under active development in several groups at universities and companies. Based on the transient formation of mechanically produced conduits in the skin, antigens are able to pass through the *stratum corneum* and taken up by the LCs and/or the DCs [15]. These microneedles should be long enough to penetrate the *stratum corneum*, but short enough to avoid the contact with the nerves in the deeper skin tissue. In this way painless vaccination *via* the skin can be achieved. Successful TCI studies with equal or higher vaccination efficacy than i.m. injection have been reported by Mikszta *et al*. applying anthrax vaccine by intradermal injection with microneedle in rabbits [16] and Widera *et al.* using ovalbumin by an antigen-coated microneedle array in hairless guinea pigs [17]. However, practically the elasticity of the skin makes effective piercing by microneedle array very difficult. For reliable piercing in human skin, arrays with microneedle lengths typically of 500 μm and longer are required [18]. So far, effective and reproducible *stratum corneum* piercing by the microneedle array remains a very important issue, which influences the dose control of dermally delivered drugs including vaccines.

We have reported that microneedle arrays manufactured from commercially available 30G hypodermal needles applied by a handheld applicator are able to enhance the transport of hydrophilic molecules with a molecular weight up to 72 kDa through dermatomed human skin *in vitro* [18]. The microneedle arrays used in that study were rather long (from 550 to 900 μm). As a consequence they might cause pain sensation and the recovery of skin barrier may take longer time. The necessity to pierce the skin reproducibly with shorter microneedle arrays is therefore obvious. Recently, in our group an electric impact insertion system has been developed which drives microneedle array into the skin at a predetermined velocity [19]. The elasticity of the skin can thus be counteracted, allowing the penetration of the shorter microneedle arrays *in vitro* into human skin. In the current study, the piercing ability of shorter microneedle arrays was validated and optimized in mouse skin *in vivo* using handheld and electric applicators before performing a TCI study, since mouse skin is thinner but more flexible than human skin.

Diphtheria is an example of a disease that is readily preventable by vaccination but remains a threat to public health due to short protection period and improper vaccination schedule. This had led to studies investigating TCI as a vaccination platform for diphtheria [20-22]. These studies revealed that dermal vaccination induced systemic response when applied with adjuvants multiple times on physically disrupted skin. In our current studies, diphtheria toxoid (DT) and influenza subunit vaccine (H3N2), average MW of 68 kDa and 228 kDa (trimmer), respectively, were both used in TCI studies aiming to examine the potential advantage of microneedle array pretreatment. Cholera toxin (CT), a well-known potent adjuvant, was co-administered to further improve the immune responses of these two vaccines. Our results show that microneedle arrays, with a needle length of 300 μm applied by the electric applicator, were able to pierce the mouse skin. It was very effective in increasing the immune response of DT. For the influenza TCI, co-administration of CT significantly improved the immune response against influenza, however, independent of microneedle array pretreatment of the skin.

Materials and methods

Materials

The human influenza vaccine is composed of the mono-valent bulk subunit vaccine A/Panama/2007/99 RESVIR-17 (H3N2, trimer, larger aggregates of trimmers exist due to the cluster of its hydrophobic trans-membrane domains) and was kindly provided by Solvay Pharmaceuticals (Weesp, The Netherlands). Antigen concentration was based on haemagglutinin content. Diphtheria toxin (DTa 79/1) and diphtheria toxoid (batch 98/40, protein content 12.6 mg/ml by BCA assay, 1 μg equal to approximately 0.3 Lf) were provided by The Netherlands Vaccine Institute (NVI, Bilthoven, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ1 chain specific) and IgG2a (γ2a chain specific) were purchased from Southern Biotech (Birmingham, US). Chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the substrate buffer were purchased from Biosource B.V (Nivelles, Belgium). O-phenyldiaminedihydro-chloride (OPD), lyophilized bovine serum albumin (BSA), Tween 20, Trypan blue and cholera toxin were purchased from Sigma (Zwijndrecht, The Netherlands). Tween 80 was purchased from Merck (Darmstadt, Germany). Nimatek® (100 mg/ml Ketamine, Euovet Animal Health B.V., Bladel, The Netherlands),

Visagel® from Eurovet, Rompun® (20 mg/ml Xylazine, Bayer B.V., Mijdrecht, The Netherlands) from Bayer and the injection fluid (0.9% NaCl) were obtained from a local pharmacy. All other chemicals used were of analytical grade and all solutions were prepared with distilled water.

Female BALB/c mice (H2d), 8-week old at the start of the experiment were purchased from Charles River (Maastricht, The Netherlands), and maintained under standardized conditions in the animal facility of the Leiden/Amsterdam Centre for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of The Netherlands.

Methods

Microneedle arrays and applicators

Three types of microneedle arrays were used in this study. First, the assembled microneedle arrays were manufactured from commercially available 30G hypodermic needles (BD, Alphen a/d Rijn, The Netherlands) as described previously [18]. The needles were assembled as a 4×4 array on a polymer plate with the surface area of around 0.5 cm^2. Serials of assembled microneedle arrays were fabricated with needle lengths of 300, 550, 700 and 900 μm (Fig. 1A). The second type was made of stainless steel wire with a diameter of about 200 μm and a length of 300 μm, with a tangentially cut tip [19]. This type will be referred to as solid microneedle array (Fig. 1B). The third one is silicon hollow microneedle arrays with a length of 245 μm, available as 4x4 and 9x9 arrays (Fig. 1C) and produced as described previously [19].

Piercing of mouse skin with microneedle arrays

The abdominal skin of the mouse was shaved 24 h prior to microneedle array treatment. Just before the treatment, mice were anaesthetized by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. The shaved skin area was wiped with gauze soaked in 70% ethanol. A skin fold of the shaved abdominal region was supported by styrofoam and pierced by microneedle arrays. The handheld applicator was pressed 1 min manually with the assembled microneedle arrays ranging from 300-900 μm microneedle length. The electric applicator was used with a velocity of 3 m/s and a retention time of 1 s for all types of microneedle arrays not longer than 300 μm [19].

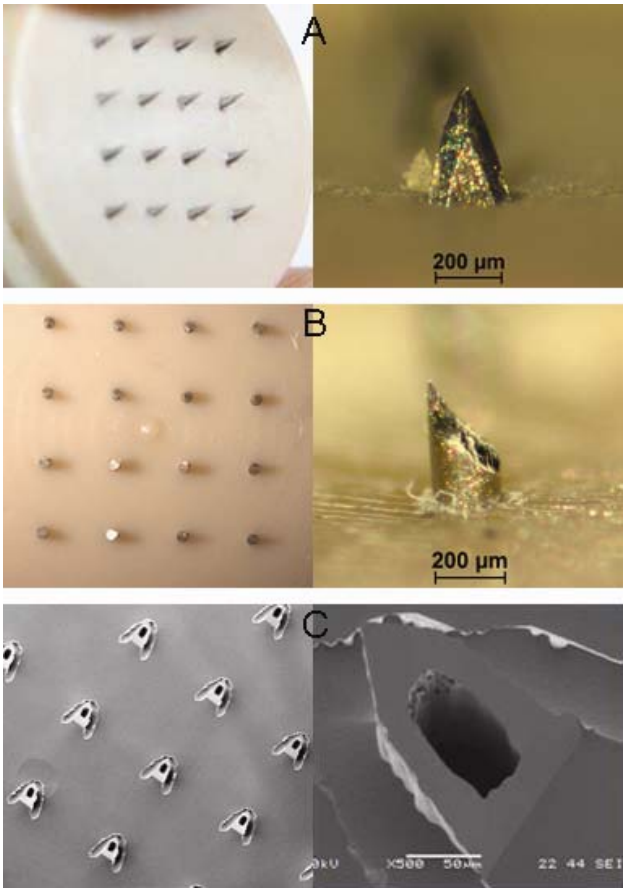

Figure 1. Three types of microneedle arrays; (A) assembled (B) solid and (C) silicon hollow microneedle arrays.

In vivo evaluation of piercing

Figure 2. Experimental setup for visualization of microneedle array piercing *in vivo*.

Immediately after piercing of the mice, the trans-epidermal water loss (TEWL) was measured by a Tewameter (TM 210, Courage + Khazaka, Köln, Germany). After placement of the probe on the pierced surface of the skin, the TEWL values were measured for 1 min and a mean value was calculated. To evaluate the uniformity of skin piercing, the site of piercing was covered with a hollow Hilltop® chamber (Miamiville, US) and filled with 300 µl 0.4% Trypan blue PBS solution for 1 h (Fig. 2). Subsequently the dye solution and chamber were removed and the mouse was sacrificed by cervical dislocation. The pierced skin was excised and examined under a magnifier. Successful

piercing of the microneedle arrays through the skin was scored by the appearance of blue dots in arrays at the dermal side of the skin.

Dermal diphtheria and influenza vaccination

The mice were shaved on their abdomen and rested for 24 h. On the day of vaccination, the mice were anaesthetized; the shaved skin area was wiped with 70% ethanol. A microneedle array was pierced into the skin using the electric applicator. For diphtheria vaccination, only the 300 µm-long solid microneedle array was investigated. Groups of 8 mice were immunized with 100 µg DT or 100 µg DT plus 100 µg CT with and without microneedle array pretreatment. DT and CT were applied on the skin in 70 µl 0.01 M citrate buffer at pH 5.0. DT formulations were carefully spread to wet the entire skin area of application (microneedle array-pretreated or untreated, ~ 2 cm^2 area restricted by a metal ring) and incubated occlusively for 1h. For the control group, 5 µg DT absorbed by $AlPO_4$ (Adju-Phos®; Brenntag Biosector, Denmark) in 100 µl saline solution was injected subcutaneously. The $AlPO_4$ adsorbed DT (DT-alum) was prepared as previously described and the adsorption of DT was between 70% and 80% [23].

For influenza vaccination, after piercing the array was withdrawn and a hollow Hilltop® chamber was attached to the skin with medical tape. The chamber was filled with 30 µg influenza haemagglutinin in 300 µl PBS (surface area around 1.2 cm^2) for 1 h incubation. Groups of 6 mice were immunized as follows: (a) PBS with solid microneedle array (300 µm-long, 4×4) treatment; (b) vaccine alone on untreated skin; (c) vaccine with silicon hollow microneedle array (245 µm-long, 4×4) treatment; (d) vaccine with silicon hollow microneedle array (245 µm-long, 9×9) treatment; (e) vaccine with solid microneedle array (300 µm-long, 4×4) treatment; (f) vaccine with 100 µg CT and solid microneedle array (300 µm-long, 4×4) treatment and (g) vaccine with 100 µg CT on untreated skin. The control group received an intramuscular injection of 15 µg influenza haemagglutinin in 100 µl physiological saline solution per mouse.

In both studies, after 1 h incubation the mice were extensively washed with lukewarm tap water, patted dry and washed again. The TCI and control groups were boosted twice using the same protocol at day 21 and day 42 (approximately the same skin region for the TCI groups), and sacrificed at day 56. Blood samples were obtained from the tail vein one day before each immunization and total blood was collected from the femoral artery under

anesthesia before euthanasia. Blood samples were collected in MiniCollect® tubes (Greiner bio-one, Alphen a/d Rijn, The Netherlands) till clot formation and centrifuged 10 min at 10,000*g* to obtain cell-free sera, then transferred to new tubes as aliquots and kept at -80 ºC until further use.

Vero cell test

The diphtheria toxin-neutralizing antibodies in mouse sera were assessed by a Vero cell test as previously described [24]. Complement in the serum was inactivated by heating at 56 ºC for 45 min. Then 2-fold serial dilutions of individual sera were prepared with complete medium 199 (CM199, Gibco, Breda, The Netherlands) and applied to microtitre plates (50 µl/well). Then, 50 µl diphtheria toxin, DTa 79/1 (0.0005 Lf/ml) in CM199, was added to each well. The plates were incubated for 2 h at 37 ºC for neutralization. Subsequently, 50 µl CM199 with 2.5×10^5 Vero cells/ml was added to each well. A reference antitoxin as well as an untreated cell control was included in each plate. The plates were covered with a plate sealer and incubated at 37 ºC in 5% CO_2 for 6 days. Each well was checked for the presence of living cells by microscope. The neutralizing antibody titer was expressed as the dilution factor of the most diluted serum that still had living Vero cells.

Serum IgG, IgG1 and IgG2a assay

The IgG subtype profile of influenza-specific antibodies was checked on day 20, 41 and 56 with ELISA as previously described [25]. Briefly, ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight at 4 °C with 200 ng/well of influenza subunit antigen (H3N2) in coating buffer (0.05 M sodium carbonate/ bicarbonate, pH 9.6). Plates were subsequently washed twice with PBS containing 0.05% Tween 20, pH 7.6 (PBST) and then blocked by incubation with 1% (w/v) BSA in PBST for 1 h at 37 °C. Thereafter the plates were washed three times with PBST. Two-fold serial dilutions of sera from individual mouse were applied to the plates and incubated for 2 h at 37 °C. Plates were then washed three times with PBST and incubated with HRP-conjugated goat antibodies against either mouse IgG1 or IgG2a for 1.5 h at 37 °C. Antibodies were detected by OPD and expressed as the reciprocal of the calculated sample dilution corresponding with absorbance of 0.2 above the background at 492 nm [26]. DT specific antibodies (IgG, IgG1 & IgG2a) and CT-specific IgG titers were determined with the same procedure by coating the plate with 140 ng DT or 66.7 ng CT per well, detecting antibody by

TMB and measuring optical absorbance at 450 nm. If a reading was above or below the relevant standard curve, further measurements were taken with lower or higher dilutions.

Hemagglutination inhibition (HI) assay

Serum samples were collected on day 20, 41 and 56 after influenza TCI. HI assay were performed at ViroClinics (Rotterdam, The Netherlands), according to WHO standard procedure using haemagglutinin antigens representing the stains of virus included in the vaccine [27]. Two-fold serial dilutions of the serum samples in PBS were incubated with the titrated antigen solution and 1% Turkey erythrocytes. The results are given as titers, meaning the highest dilutions of the serum which achieves complete inhibition of hemagglutination. All sera were titrated simultaneously in duplicate. Detection limit of this assay was 10 and non-responding sera were assigned an arbitrary titer of half the detection limit.

Statistical analysis

Statistical comparisons were made using two-tailed unpaired Student's *t*-test or one way ANOVA with Tukey post test where suitable using the software Prism Graphpad. A *p* value less than 0.05 was considered to be significant.

Results

In vivo evaluation of piercing in mouse skin

In the first set of experiments, the piercing capacity of microneedle arrays *in vivo* on mouse skin was evaluated using assembled microneedle arrays (300 to 900 μm long) and the handheld applicator. Figure 3a shows a clear trend between the TEWL and the needle length of the microneedle arrays, and demonstrated higher TEWL than those of the untreated control.

The 4x4 microneedle arrays with a needle length > 550 μm formed patterns of blue dots on both the *stratum corneum* side and the dermal side of the skin while no blue spots could be observed after application of 300 μm-long ones (Fig. 3b). This clearly indicates that microneedle with needle lengths of 550 μm and longer applied manually pierced the mouse skin and created conduits, while the 300 μm-long one did not.

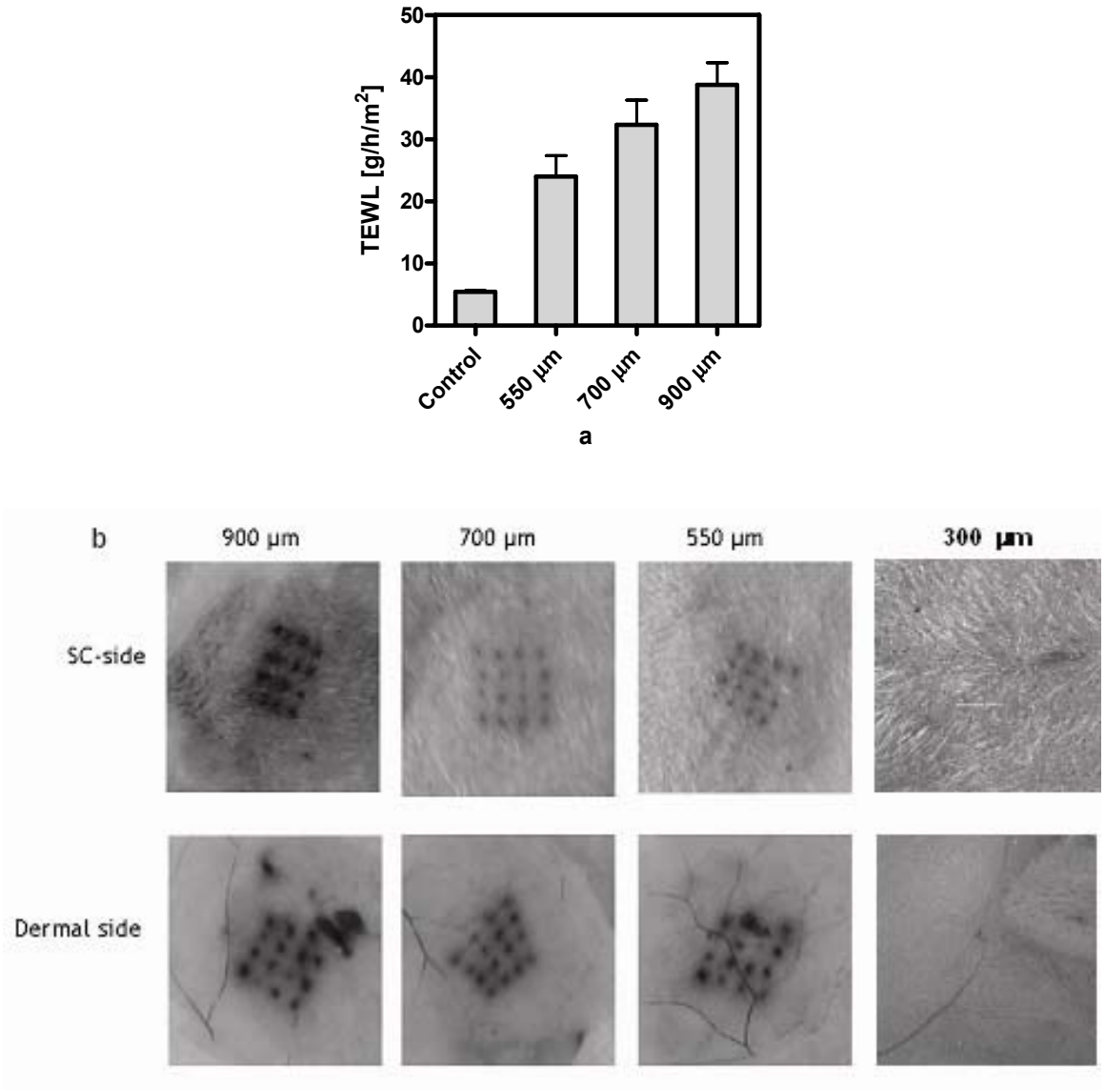

Figure 3. Assembled microneedle arrays with needle length of 300, 550, 700 and 900 µm were applied by the handheld applicator on mouse skin *in vivo*; (a) TEWL before and after microneedle arrays; (mean + SD, n=3). (b) Trypan blue staining. Pictures were taken from the stratum corneum and the dermal side of excised mice skin.

As manual piercing of 300 µm-long microneedle was not successful, it was decided to optimize the piercing of shorter microneedle array (*i.e.* ≤ 300 µm) by applying the array at an adjustable speed. For this an electric applicator was designed. A proper velocity and the sharpness of needles were expected to counteract the elasticity of the skin and pierce the *stratum corneum* more effectively as was already demonstrated in human skin [19]. Immediately after piercing, TEWL was measured (Fig. 4a). Elevated TEWL compared to the

control is consistent with the results obtained by the Trypan blue staining, namely short microneedles were able to pierce the skin when projected at the speed of 3 m/s. For all microneedle arrays including 245 µm-long silicon ones (4x4 and 9x9), blue spot arrays were clearly visible at the dermal side of the skin (Fig. 4b), indicating that piercing of all tested microneedle arrays was successful. Therefore the short microneedle arrays and the electric applicator were chosen for the *in vivo* vaccination studies.

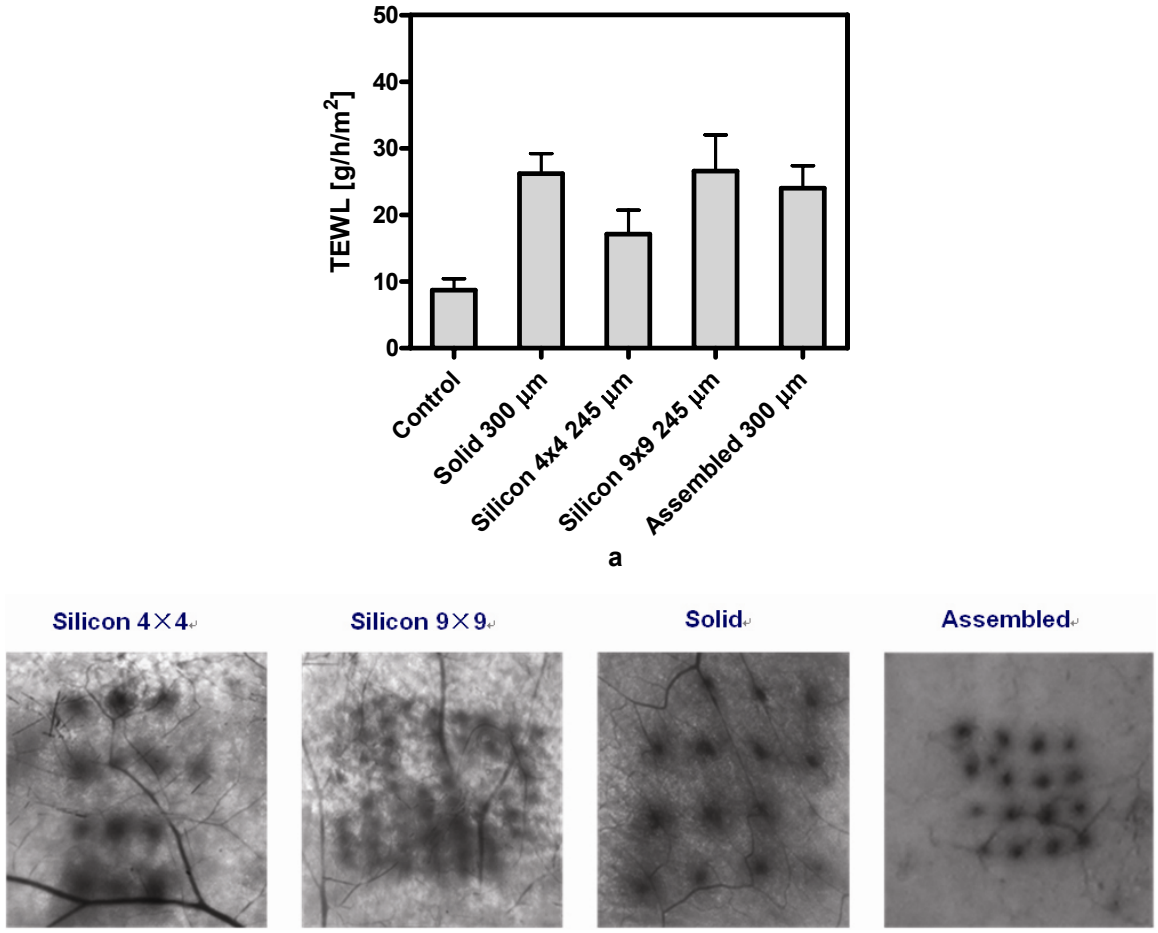

Figure 4. 245 µm-long silicon microneedle arrays (4×4 and 9×9), 300 µm-long solid and 300 µm-long assembled microneedle arrays were applied by the electric applicator on mouse skin *in vivo*; (a) TEWL before and after microneedle arrays treatment (mean ± SD, n=3); (b) Trypan blue staining after treatment. Pictures were taken from the dermal side of excised mice skin.

Dermal diphtheria vaccination

During the *in vivo* studies, there was no adverse effect from the shaving, anesthetizing, piercing, immunization, or washing procedure observed.

Neither erythema nor induration was seen at the immunization site after the antigen exposure.

TCI of DT was performed with or without microneedle array pretreatment, in the presence or absence of CT as adjuvant. Plain DT applied on intact skin induced low IgG titers. However, when DT was applied on microneedle pretreated skin, titers of all IgG subtypes were increased significantly (Fig. 5a, $p<0.01$). The presence of CT on microneedle pretreated skin (after prime, the 1st boost and the 2nd boost) induced respectively 4, 20 and 4 fold higher IgG titers than those without CT. However, CT made less significant difference in DT-specific IgG titers on untreated skin. IgG levels of all TCI groups increased after each boost, while in the group of CT with microneedle pretreatment, IgG titers reached the maximum level after the 1st boost and remained at a plateau after the 2nd boost. IgG titers of this group showed the same level as compared to the group with DT-alum injection. The ratio of IgG1 to IgG2a titers indicate whether the immune responses are biased towards T helper type 1 (Th1) or T helper type 2 (Th2). Th2 biased immune responses were found in the TCI groups with microneedle pretreatment where similar IgG1/IgG2a ratios to the injection group were detected (Fig. 5b). It appears that the CT lowered the IgG1/IgG2a ratio and therefore skewed the immune response to Th1 direction. Without microneedle array pretreatment, no protective effect was induced from TCI on intact skin in the presence and absence of CT as indicated from the Vero cell test (Fig. 5c). However, with microneedle pretreatment, CT did stimulate the production of toxin-neutralizing antibodies. Serum samples from the group with CT and microneedle pretreatment contained similar toxin-neutralizing capacity compared to that of the DT-alum injection group ($p>0.05$).

CT-specific IgG titers were also checked in this context to determine whether CT itself or its subunits were transported across the skin (Fig. 6). CT is a very immunogenic antigen and the IgG titer reached very high level after prime independent of microneedle array treatment and remained at a plateau. This demonstrates that CT or its subunits were transported across the *stratum corneum* even in the absence of microneedle array treatment.

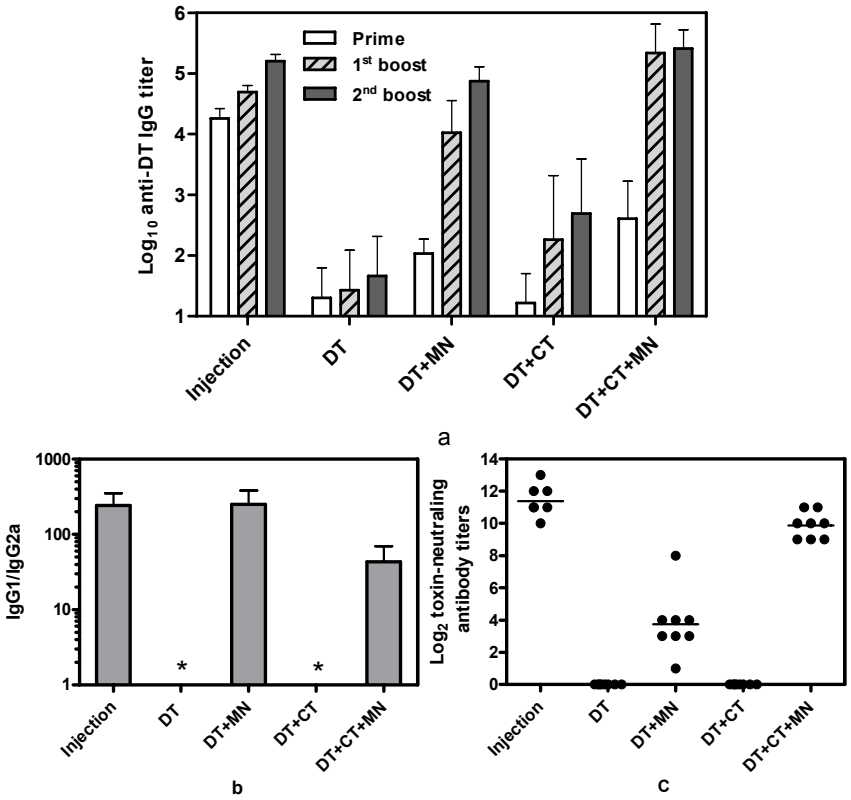

Figure 5. Immune responses in mice (n=8) after DT TCI with microneedle array (300 μm-long solid, 4×4) pretreatment on day 0, 21 and 42. (a) DT-specific serum IgG titer measured on day 20, 41, and 56. Non-responders were given an arbitrary value of 1. (b) DT-specific serum IgG1 and IgG2a titers determined on day 56. Ratios of IgG1/IgG2a titers of individual mouse were shown as mean + SD (*IgG2a titers below detection limit). (c) Neutralizing antibody titers of individual mouse, as measured with Vero cell test. Data shown are the dilution factor of the most diluted serum sample which protects Vero cells to survive incubation with diphtheria toxin. (MN=microneedle array pretreatment)

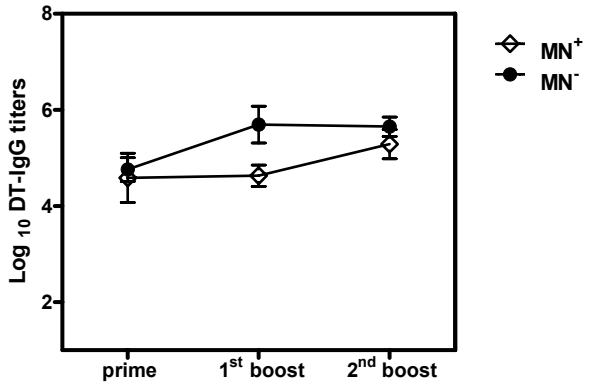

Figure 6. CT-specific serum IgG responses in mice (n=8) following the immunization by 100 μg of DT and CT on day 0, 21 and 42. Antibodies were measured on day 20, 41, and 56 and data displayed as mean ± SD. (MN=microneedle array pretreatment)

Dermal influenza vaccination

HI assay determines the titer of functional antibodies, by measuring the inhibition of red blood cell agglutination by influenza virus. In the influenza TCI study, non-adjuvanted vaccine on intact skin induced low HI titers. Different from DT TCI, no improvement was achieved by the application of any microneedle arrays (Fig. 7a). However, when CT was co-administrated as adjuvant, it drastically increased the immune response to influenza vaccine, independent of microneedle pretreatment. The presence of CT resulted in higher HI titers than in the absence of CT, around 8 fold after prime, 32 fold after the 1st boost and 8 fold after the 2nd boost, respectively. A similar trend was observed in IgG subtype titers as compared to HI titers (Fig. 7b & 7c). No differences in HI and IgG subtype titers were indicated among the groups with treatment of different microneedle arrays and the group without treatment.

Discussion

The objective of this study was to evaluate microneedle array treatment in TCI of DT and influenza vaccine as a potential alternative method for injection. The benefit from using microneedle arrays are: i) increase in antigen transport across the skin barrier due to the created conduits, ii) increased targeting to DCs in the dermis as the conduits can reach the upper part of the dermis [19] and/or iii) activation of the LCs and DCs, which result in an increased immune response. Recently, microneedle arrays have attracted considerable attention in the application of dermal vaccination. Studies including dermal administration of influenza vaccine, plasmid DNA encoding hepatitis B surface antigen and recombinant protective antigen of *Bacillus anthracis* resulted in immune responses at least as strong as those generated by subcutaneous or intramuscular injection [16, 28, 29]. Influenza intradermal vaccination using hollow microneedle has shown dose-sparing and been proven to be safe in human clinical trials [30]. Further application of microneedle array in dermal vaccination and transdermal drug delivery is promising.

When using microneedle arrays, the microneedles should pierce the skin reproducibly without any pain sensation. Recently, skin irritation, pain sensation and skin barrier function were examined using microneedle arrays with a length varying between 200 and 550 μm *in vivo* in human volunteers using the same electric applicator as used in this study. The microneedle

arrays caused only minimal pain sensation and skin irritation, while a reduction in skin barrier function was observed [31]. These properties make the microneedle array and its applicator an excellent candidate delivery device for TCI.

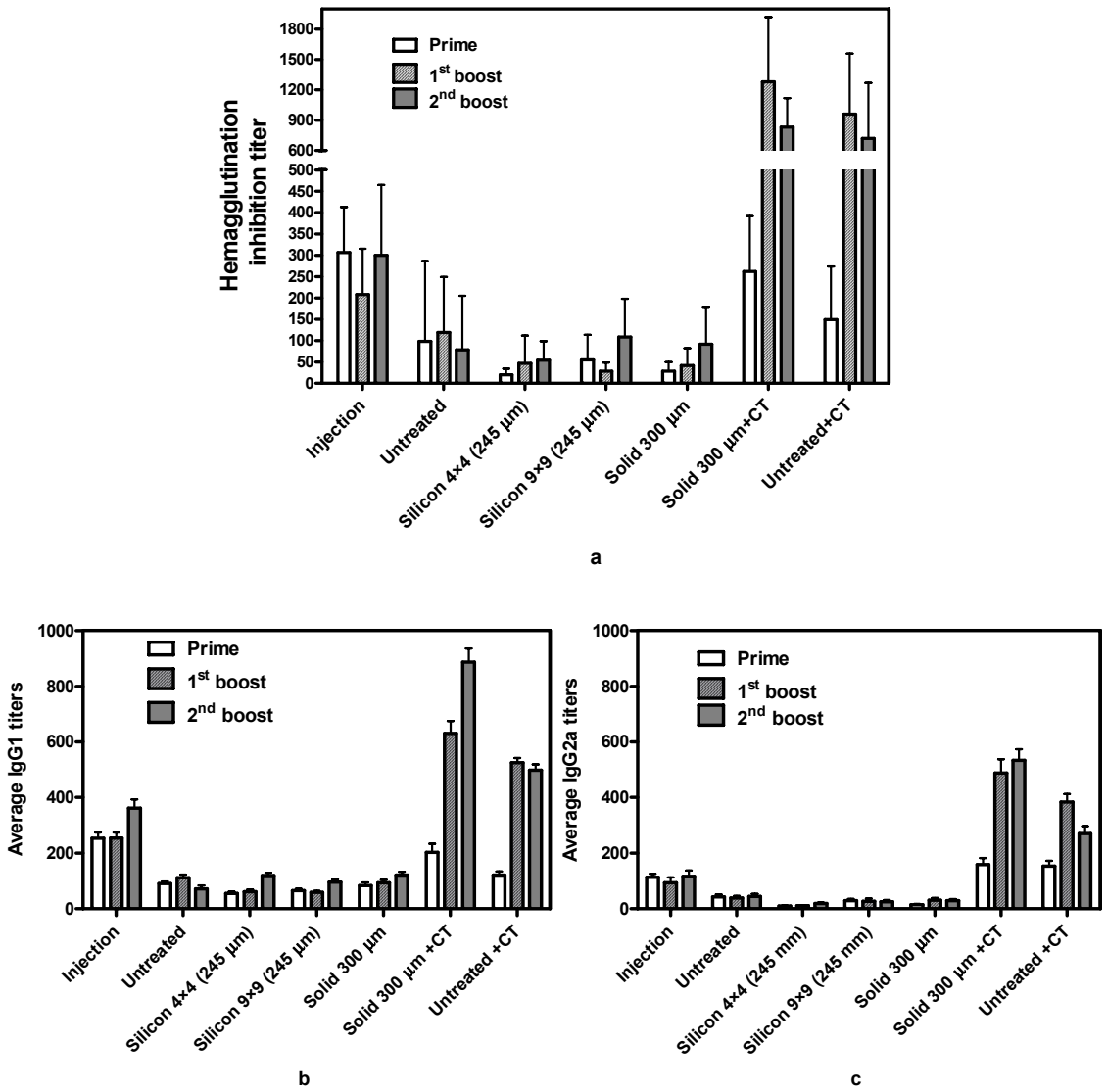

Figure 7. Immune responses in mice (n=6) after influenza TCI with microneedle arrays pretreatment on day 0, 21 and 42. (a) Hemagglutination inhibition was tested against influenza virus (H3N2) on day 20, 41 and 56 using Turkey red blood cells. Data displayed as the dilution factor of the most diluted serum sample that inhibits influenza agglutination (mean + SD), non-responding sera were assigned an arbitrary titer of 5, half of the detection limit. (b) & (c) Serum IgG1 & IgG2a titers against influenza subunit antigen (H3N2) were measured with ELISA on day 20, 41 and 56 and shown as mean + SD.

Piercing mouse skin

The first part of this study was to examine and validate the *in vivo* piercing capacity of various microneedle arrays (the assembled, solid and silicon hollow arrays with different needle lengths) using both the handheld and the electric applicators. The low TEWL values measured before microneedle array treatment but after shaving indicated that the shaving and the 70% ethanol wipe did not increase the permeability of the skin and the integrity was well preserved (Fig. 3 and 4). With the handheld applicator, piercing was only possible with 550 μm and longer microneedles. By using the newly designed electric applicator and driving microneedle arrays at the speed of 3 m/s, 300 μm and even 245 μm long microneedles pierced mouse skin *in vitro* and *in vivo* in a reproducible manner, similar as observed in human skin *in vitro* [19]. This is an important observation considering that mouse skin is much more flexible than human skin and demonstrates that piercing with an optimal velocity effectively counteracts the elasticity of the mouse skin.

Comparison of dermal DT and influenza immunization

Microneedle array pretreatment

Through TCI on intact skin, neither DT nor influenza vaccine induced substantial immune responses as indicated by low level of IgG and functional antibody titers. During TCI after microneedle array pretreatment, the vaccine has to diffuse along the conduits into the skin. However, only a fraction of the total dose may enter these conduits. Microneedle array pretreatment provided major improvement as compared to plain DT applied on intact skin, and with the presence of CT, it reached similar levels of serum IgG and toxin-neutralizing antibody titers as those of the DT-alum injection group. TCI with influenza presented a different result as compared to DT. The immune responses induced by plain vaccine were very low as indicated by the HI and IgG titers when compared to CT-adjuvanted vaccine, independent of microneedle array pretreatment. Several reasons may account for this result. i) Lower potency of the subunit influenza vaccine as compared to DT; ii) Lower concentration of vaccine applied (0.1 μg/μl influenza vaccine *vs.* 1.45 μg/μl DT); iii) The aggregates formed by the hydrophobic transmembrane domains of haemagglutinin trimmers may further reduce its permeation. The relatively low immune responses induced by plain influenza vaccine in barrier-disrupted skin were also reported by Garg *et al*. who vaccinated mouse with subunit

influenza vaccine (H5N1) using the Passport™ system (a patch device creating pores by thermal ablation) [8] and Skountzou *et al*. who applied inactivated influenza virus (WIV, PR8) on tape-stripped mouse skin pretreated with 70% ethanol [32].

Adjuvanticity of CT

In the current study, the presence of CT played an important role in enhancing both immune responses. It was previously shown that CT is toxic when administered mucosally, while it does not exert any toxicity when applied transcutaneously [33]. From the results described here, the adjuvanticity of CT on dermal delivered vaccines is antigen dependent. It was shown by the Vero cell test that CT, as an adjuvant in DT dermal vaccination, failed to stimulate protective responses to diphtheria toxin when co-administered on intact skin. However, high toxin-neutralization capacity could be achieved when applying DT and CT on microneedle array pretreated skin. CT-adjuvanted influenza TCI induced substantially higher immune responses concerning HI as well as IgG subtype titers in the serum, which were much higher than those after intramuscular injection of plain vaccine. Therefore, the addition of CT may lead to dose sparing benefit for influenza TCI. Furthermore, one should be aware of the difference in efficiency of TCI when comparing the murine model with human. The mouse skin has much thinner *stratum corneum* with considerably higher density of hair follicles than human skin. The relative area of application in mice is much larger than in humans. TCI in mice, therefore, can be successful even without physical pretreatment of the skin for certain antigens. However, when moved to humans, it is more difficult to get a protective response [34]. Potent adjuvant as well as microneedle array pretreatment may be necessary to archive effective protection and dose sparing.

Antigen penetration through the skin barrier

CT is well known as a potent antigen for dermal vaccination which actually initiated the research on TCI [35]. The current study showed clearly that CT, as an antigen with MW of 84 kDa, induced high levels of CT-specific IgG titers without increasing skin permeability. The response to TCI of influenza vaccine does not rely on microneedle array pretreatment, either. In general it is stated that when molecules are larger than 500 Da, the skin penetration reduces drastically [36]. Although TCI of vaccine and adjuvant is intended for delivery to the more superficial epidermis, it is remarkable that CT and influenza

vaccine or their subunits can penetrate the *stratum corneum* different from DT. We speculate that the vaccines may diffuse through the hair follicles as it has been shown that particles up to 40 nm can diffuse through the hair follicular opening and reach epidermal LCs [37, 38]. Taking the data of influenza and DT vaccination together, microneedle array pretreatment and CT co-administration seem to involve different diffusion pathway and immunological mechanisms. The fact that CT is a more potent adjuvant for influenza subunit vaccine than for DT may be due to the properties of the individual antigen, *e.g.* pathology of the disease, antigenicity of the vaccine, charge of the vaccine molecule, particular status of the vaccine and the molecular interactions between antigen and CT. It is not fully understood and requires more investigation.

In summary, we showed in this study that TCI can be strongly improved by using microneedle arrays combined with adjuvant. For DT, the microneedle array pretreatment is essential to obtain high antibody response, which is modestly augmented further by co-administration of CT. TCI of DT and CT after microneedle treatment results in comparable protection as injection of DT-alum. In contrast, for influenza vaccine, CT is very efficient in potentiating the immune response but does so independent of microneedle array pretreatment of the skin. TCI of influenza vaccine with CT is superior to the injection method.

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Chapter 4

Immune modulation by adjuvants combined with diphtheria toxoid administered topically in BALB/c mice after microneedle array pretreatment

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Introduction

Currently, most vaccines are administrated by injection. Costs, risks and discomforts associated with the use and abuse of needles have boosted research on needle-free vaccinations [1]. Around ten years ago, Glenn *et al.* reported for the first time data on transcutaneous immunization (TCI) and showed that strong immune responses could be induced by topically applied cholera toxin [2]. TCI is particularly attractive because of the high accessibility of the skin and the presence of antigen-presenting cells (APCs) in the epidermis and dermis, in particular the Langerhans cells (LCs) and the dermal dendritic cells (DCs) [3]. However, the upper layer of the skin, the *stratum corneum*, acts as a barrier for diffusion of macromolecules and therefore is a major obstacle to dermal vaccine delivery. To overcome this barrier and achieve effective TCI, physical methods such as intradermal injection [4, 5], thermal ablation [6], microdermabrasion [7] electroporation [8] and cavitational ultrasound [9] have been used. Physical disruption of the skin barrier increases the percutanous penetration of the antigen and makes the antigen more readily available for sampling by APCs [10]. Moreover, disruption of the skin barrier may induce a chain of molecular events that lead to the secretion of pro-inflammatory cytokines and facilitate APC activation.

A relatively novel approach to disrupt the skin barrier in a controlled manner with little pain sensation is the use of microneedle arrays. It was proposed first by Gerstel and Place already in the 1970s [11]. Ten years ago, when the technology for fabrication in micron dimensions became readily available, Prausnitz resumed the study using microneedle arrays in transdermal drug delivery [12]. When used as a pretreatment, microneedle arrays enable antigens to diffuse along the transiently formed tiny conduits through the *stratum corneum*. Thereby antigens may be able to approach the LCs in the epidermis and the DCs in the dermis [13]. Using an ovalbumin-coated microneedle array, Matriano *et al.* evaluated the uniformity of skin piercing, and studied the dose of the vaccine used and the kinetics and magnitude of antibody titers induced in hairless guinea pigs [14]. Widera *et al*. investigated the influence of important fabrication parameters, *e.g.* length and density of the microneedles, area and coating of the microneedle arrays, on vaccination efficiency of TCI [15]. Hooper *et al.* reported that smallpox DNA vaccine-coated microneedle arrays applied topically in combination with electroporation protected mice against lethal challenge [16]. Recently, Van Damme *et al.* tested the injectable microneedle array in human volunteers

using influenza vaccine, resulting in a comparable seroprotection rate as compared to i.m. injection with 5 fold dose sparing [17]. These interesting results show that TCI using microneedle arrays is promising. However, after more than ten years of extensive research [18, 19], there continues to be a need for further improvement of microneedle array mediated TCI, *e.g.* by using potent adjuvants or novel ways of applying the microneedle arrays.

Recently a new electric applicator was developed in our lab. It is designed to insert microneedle arrays into the skin with a predetermined velocity and thereby counteracts the elasticity of the skin. This applicator enables us to reproducibly pierce human and mouse skin *in vivo* with microneedles with a length of 300 µm or less [18]. DT was recruited as a model antigen to evaluate the potential of microneedle array pretreatment in TCI. We mentioned in Chapter 3 that the immunogenicity of topically applied DT was dramatically improved by microneedle array pretreatment as compared to untreated skin.

Table I. Properties of the adjuvants employed in the current study (adapted from [19-21])

Adjuvant	Type	Cell-mediated immunity (Th1)	Humoral immunity (Th2)	Receptor
Cholera toxin	Exotoxin	+[1]	+++	GM1 ganglioside
lpxL1 LPS	Endotoxin	++	++	TLR4
Quil A	Saponin based	+++	++	Not identified
CpG	Bacterial DNA	++++	+	TLR9
Alum	Inorganic salt	+	+++	Not identified

[1] Humoral and cellular immunity in arbitrary units represent the ability of adjuvants to enhance Th2 response or Th1, respectively, to foreign antigens.

The objective of the present study was to determine the effect of adjuvants on the quantity and quality of the immune response against DT after TCI on microneedle-pretreated skin. The Th1/Th2 balance of the immune response depends on several factors including the nature of the antigen and the adjuvant, the delivery route and the targeted APCs, as suggested by the ratio of IgG1/IgG2a antibody titers [19]. The adjuvants included in this study, cholera toxin (CT), lipopolysaccharide (LPS), synthetic oligo deoxynucleotide containing a CpG motif (CpG), immunostimulatory fractions extracted from the bark of the tree *Quillaja saponaria* (Quil A) and aluminum phosphate

(alum), differ in their adjuvant mechanism and ability to modulate the immune response (see Table I) [19]. Immune modulation by these adjuvants was evaluated in TCI on microneedle-pretreated skin and compared with conventional subcutaneous (s.c.) injection of DT or DT-alum, by measuring serum IgG (subtype) titers and neutralizing antibody titers.

Materials and methods

Materials

Diphtheria toxin (batch 79/1), diphtheria toxoid (batch 98/40, protein content 12.6 mg/ml by BCA assay, 1 µg equal to approximately 0.3 Lf) and the *lpxL1* LPS were provided by the Netherlands Vaccine Institute (NVI, Bilthoven, The Netherlands). Horseradish peroxidase-conjugated goat anti-mouse (HRP-GAM) IgG (γ-chain specific), IgG1 (γ1-chain specific) and IgG2a (γ2a-chain specific) were purchased from Southern Biotech (Birmingham, USA). Quil A and Adju-Phos® (alum) were obtained from Brenntag Biosector (Copenhagen, Denmark). CpG oligo deoxynucleotide 1826 (5'-tcc atg acg ttc ctg acg tt-3', phosphorothioated) was synthesized by Isogen Biosolutions (IJsselstein, The Netherlands). Chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the substrate buffer were purchased from Biosource B.V. (Nivelles, Belgium). Cholera toxin was ordered from Sigma-Aldrich (Zwijndrecht, The Netherlands). Nimatek® (100 mg/ml ketamine, Euovet Animal Health B.V., Bladel, The Netherlands), Rompun® (20 mg/ml xylasine, Bayer B.V., Mijdrecht, The Netherlands) and the injection fluid (0.9% NaCl) were obtained from a local pharmacy. All other chemicals used were of analytical grade and all solutions were prepared with distilled water.

Animals

Female BALB/c mice (H2d), 8-week old at the start of the experiment were purchased from Charles River (Maastricht, The Netherlands), and maintained under standardized conditions in the animal facility of the Leiden/ Amsterdam Center for Drug Research, Leiden University. The study was conducted in conformity with the Public Health Service Policy on use of laboratory animals and had been approved by the Research Ethical Committee of Leiden University (UDEC, nr. 07016).

Methods

Microneedle array and applicator

Assembled microneedle arrays were manufactured from commercially available 30G hypodermic needles (BD, Alphen a/d Rijn, The Netherlands) as described previously [13]. The needles were assembled as a 300 µm-long, 4×4 array on a polymer back plate with a surface area of about 0.5 cm^2. The electric applicator was developed and optimized as mentioned in Chapter 3. The microneedles were pierced into mouse skin using a velocity of 3 m/s.

Immunization study

The DT-alum formulations were prepared as previously described and the adsorption of DT to alum was between 70% and 80% [22]. As control groups, 5 µg of DT (~1.5 Lf) with and without alum in 100 µl solution/suspension was administered per mouse by s.c injection. The other vaccine-adjuvant formulations were freshly prepared by mixing DT and the adjuvants in buffer solution in appropriate amounts as indicated in Table II. 100 µg DT and adjuvants per mouse were applied on intact or microneedle-pretreated skin as described in Chapter 3. A mutant of LPS, *lpxL1* LPS, with reduced toxicity but retained adjuvanticity, was employed in this study [23]. The dose of alum and *lpxL1* LPS used in TCI were based on the w/w ratio of antigen/adjuvant used in the injection control group and a previous immunization study, respectively [23].

During vaccination, mice were anaesthetized by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. For all groups receiving transcutaneous vaccination, the abdominal skin of the mice, shaved 24 h prior to vaccination, was first wiped with 70% ethanol. For TCI group with microneedle pretreatment, a skin fold was supported by styrofoam and pierced using the microneedle array and the electric applicator. Then 70 µl DT-adjuvant formulation was carefully spread to wet the entire skin area of application (microneedle array-pretreated or untreated, ~2 cm^2 area restricted by a metal ring). After 1 h of occlusive incubation, the skin area was extensively washed with lukewarm tap water and patted dry twice. All mice were immunized three times on day 1, 21 and 42 (at approximately the same skin region for all the TCI groups) and sacrificed on day 56. Blood was sampled from the tail vein one day before each immunization and the whole

blood was collected from the femoral artery during sacrifice. Cell free sera were obtained using MiniCollect® tubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) by centrifugation after clot formation and stored at -80 ºC.

Table II. Formulations prepared for TCI study

DT Dose (µg)	Adjuvants	Adjuvant Dose (µg)	Solvent (pH)	Volume (µl)
For s.c. injection				
5	$AlPO_4$	150	0.9% Saline (7.0)	100
5	--	--	0.9% Saline (7.0)	100
For TCI				
100	--	--	PBS[1](7.4)	70
100	*lpxL1* LPS	25	PBS/Tris[2] (7.4)	70
100	Quil A	100	PBS (7.4)	70
100	CT	100	PBS (7.4)	70
100	CpG	100	PBS (7.4)	70
100	$AlPO_4$	3000	0.9% Saline (7.0)	70

[1] PBS: 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.06 mM $Na_2HPO_4 \cdot 7H_2O$
[2] PBS/Tris: PBS mixed with 1 mM Tris-HCl (v/v=5:3)

Serum antibody assay

Serum IgG, IgG1 and IgG2a titers were determined with ELISA as previously described [24]. Briefly, ELISA plate (Microlon®, Greiner Bio-one, Alphen a/d Rijn, The Netherlands) wells were coated with DT at 4°C overnight. Two-fold serial dilutions of serum samples were applied in the plates and the containing DT-specific antibodies were detected by HRP-GAM IgG, IgG1 or IgG2a using TMB as substrate. Antibody titers are expressed as the reciprocal of the calculated sample dilution corresponding to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance-log dilution curve. If samples were not diluted in the optimal range, additional measurements were performed to generate an s-shaped curve. Subsequently, the titers were calculated using a four-parameter fitting of the curve. Samples that did not reach the half-saturated absorbance value at the lowest (ten fold) dilution were considered as non-responders.

Neutralizing antibody assay

Immunity against diphtheria depends on the presence of circulating toxin-neutralizing antibodies. These antibodies were evaluated using Vero cell test, the WHO standard method to assess the success of diphtheria vaccination, which relies on the inhibition of a cytotoxic dose of diphtheria toxin [24]. In brief, after complement inactivation, two-fold serial dilutions of serum samples were prepared with complete medium 199 (CM199, Gibco, Breda, The Netherlands) and applied to microtiter plates (CELLSTAR®, Greiner Bio-one, Alphen a/d Rijn, The Netherlands). Subsequently, 2.5×10^{-5} Lf diphtheria toxin was added to the wells. After 2 h incubation at 37 ºC for neutralization, Vero cells suspension in CM199 was added to each well. Covered with a plate sealer, Vero cells were incubated at 37 ºC in 5% CO_2 for 6 days. The end point was taken as the highest serum dilution protecting the Vero cells.

Statistical analysis

ELISA titers were logarithmically transformed for better normality before statistical analysis. Two-way ANOVA with Bonferroni posttest, one way ANOVA with Tukey posttest, or Kruskal-Wallis nonparametric test with Dunns posttest were performed as indicated. Statistical analysis was carried out using Prism (Graphpad, San Diego, USA) and a *p* value less than 0.05 was considered to be significant.

Results

During the immunization study, there was no adverse effect from the shaving, anesthesia, piercing, immunization, or washing procedure observed. Neither erythema nor induration was seen at the immunization site after exposure of antigen and adjuvants.

Immune response improved by microneedle array pretreatment

The IgG titers of mice from all groups after prime, 1st boost and 2nd boost (day 20, 41 and 56) are shown in Fig. 1a-e and an overview of the DT-IgG titers after the 2nd boost is shown in Fig. 1f. It is clear that non-adjuvanted DT did not result in a substantial IgG response when applied on intact skin (TCI). Microneedle array pretreatment provided major improvement to the immunogenicity of DT. After prime, more mice responded with higher mean

IgG titers in the presence of microneedle array pretreatment in the TCI groups. After the 1st boost, all mice of those groups responded and the IgG titers were 100 fold higher as compared to TCI on intact skin (p<0.01) and the difference of IgG titers was further increased to over 1000 fold after the 2nd boost (p<0.001, Fig. 1a). Immunogenicity of DT was further improved by the co-administered adjuvants *e.g.* Quil A, CpG and CT. The improvement by the adjuvant in question is indicated by the space in between the black and the brown lines in Fig. 1b, 1c, 1d and 1e, respectively. DT-specific IgG titers in the CT group after the 2nd boost were the highest among all TCI groups and reached levels very close to those from DT-alum injection group (p>0.05). For the adjuvants in the absence of microneedle array pretreatment, their effects on the DT immunogenicity were more variable and less pronounced. Only CT moderately improved the IgG titers after the 2nd boost (Fig. 1a and 1e, p<0.05), while the presence of the other adjuvants did not augment the IgG titers significantly. Comparing the two injection control groups, alum speeded up IgG development and increased the IgG titers significantly (p<0.001 after 1st boost). However, when DT-alum was applied on microneedle-pretreated skin, no adjuvanticity was observed as the IgG titers were lower than those of plain DT (data not shown).

Immune modulation by adjuvants in TCI on microneedle-pretreated skin

Focusing on the TCI with microneedle pretreatment, immune modulation functions of the adjuvants were investigated. The DT-specific IgG1 and IgG2a titers in the sera collected after prime, the 1st boost and the 2nd boost (day 21, 42 and 56) were determined (Fig. 2). For all adjuvants, IgG1 showed similar titers as compared to the total IgG titers and each vaccination showed a booster effect. After the 1st boost, Quil A and CT enhanced the IgG1 titers significantly as compared to plain DT (p<0.01 and 0.001, respectively). After the 2nd boost, only CT significantly enhanced the IgG1 titers as compared to plain DT (p<0.01). A clear effect of the adjuvants on the IgG2a titers was observed after the 2nd boost, which increased in the following sequence: plain DT, LPS, Quil A, CT and CpG.

The IgG1/IgG2a ratio, which is considered a measure for the Th1/Th2 balance of the immune response [25], of individual mice from each group after the 2nd boost was calculated and presented in Fig. 3. Plain DT and DT-alum induced an Th2 biased response when delivered *via* s.c. injection. Applying plain DT by the microneedle-mediated TCI induced the same IgG1/IgG2a

ratio as by s.c. injection. With adjuvants, the ratio decreased in the following sequence: plain DT, Quil A, CT and CpG, suggesting that the Th2 biased immune response induced by plain DT was skewed towards the Th1 direction.

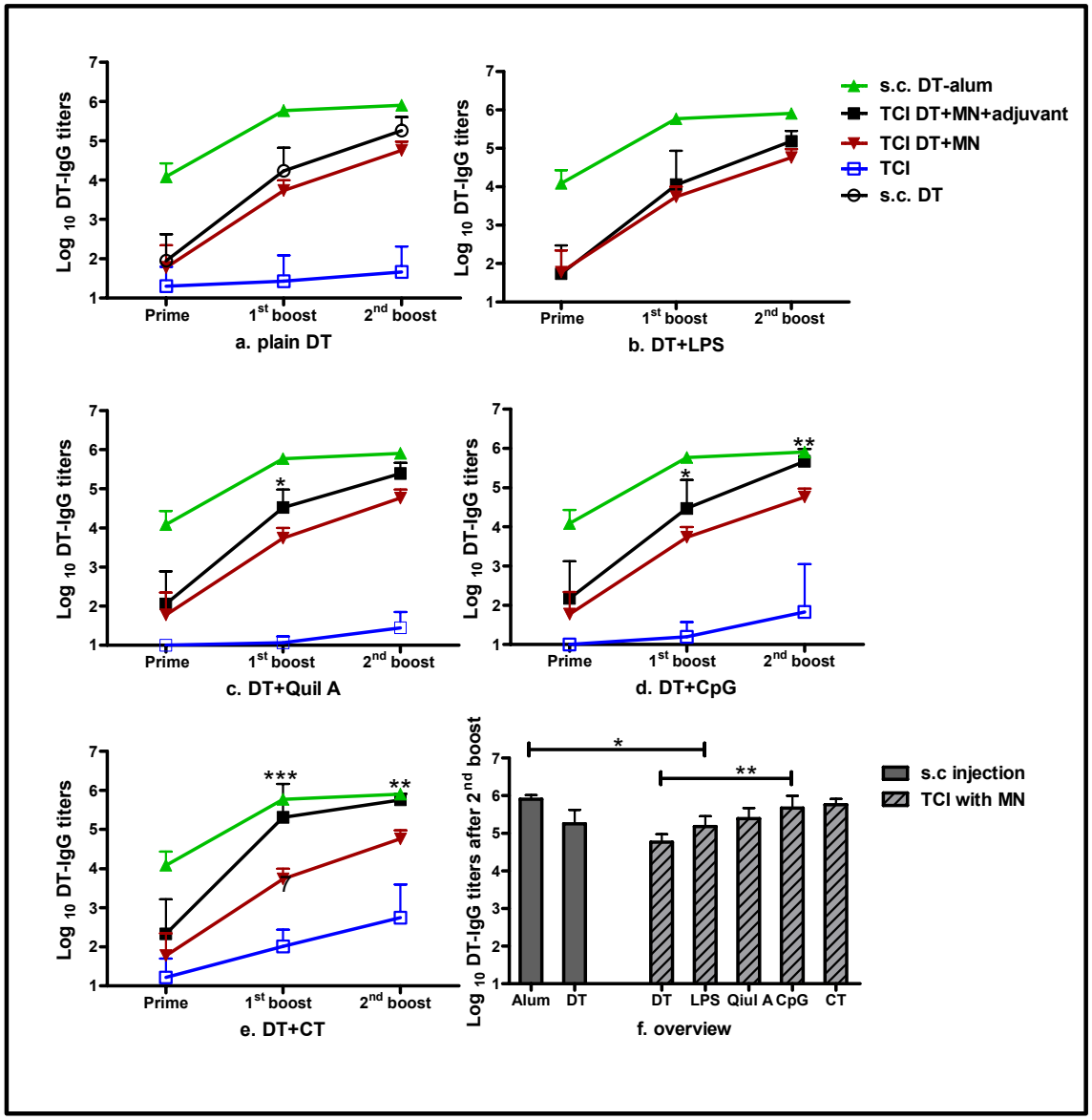

Figure 1. DT-specific IgG titers to transcutaneously delivered DT with and without microneedle pretreatment of the skin: (a) Plain DT; (b) DT + LPS; (c) DT+ Quil A; (d) DT + CpG; (e) DT + CT. Sera were collected after prime, the 1st and 2nd boost (day 20, 41 and 56) and antibodies were determined with ELISA. TCI with DT+LPS was not carried out. An overview of the DT-specific IgG titers after the 2nd boost from the microneedle-mediated TCI are shown in (f). Data are shown as mean + SD (n=8). Non-responders were given an arbitrary titer of 10. (MN=microneedle array pretreatment, * p<0.05, ** p<0.01, *** p<0.001, two way ANOVA with Bonferroni posttest)

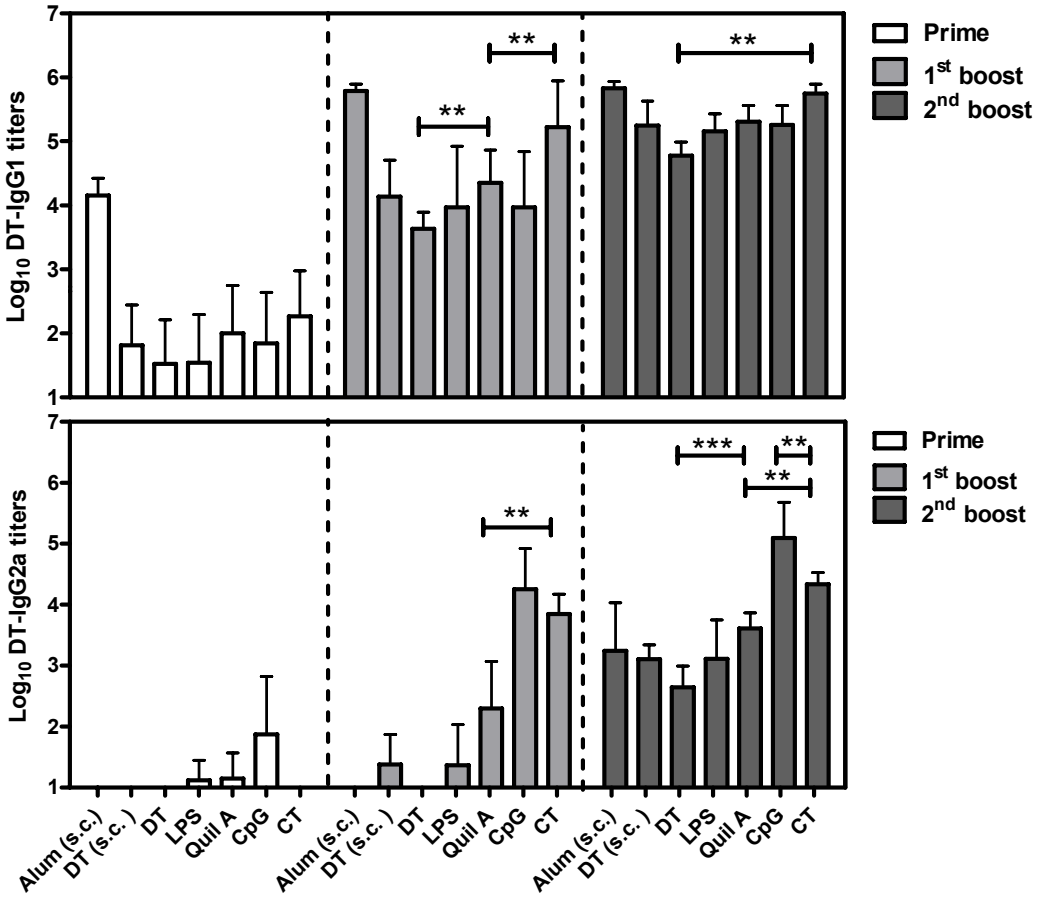

Figure 2. DT-specific IgG1 and IgG2a titers. Sera were collected after prime, the 1st and 2nd boost (day 20, 41 and 56) and antibodies were determined with ELISA. Data shown as mean + SD (n=8). Non-responders were given an arbitrary titer of 1. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, two way ANOVA with Bonferroni posttest)

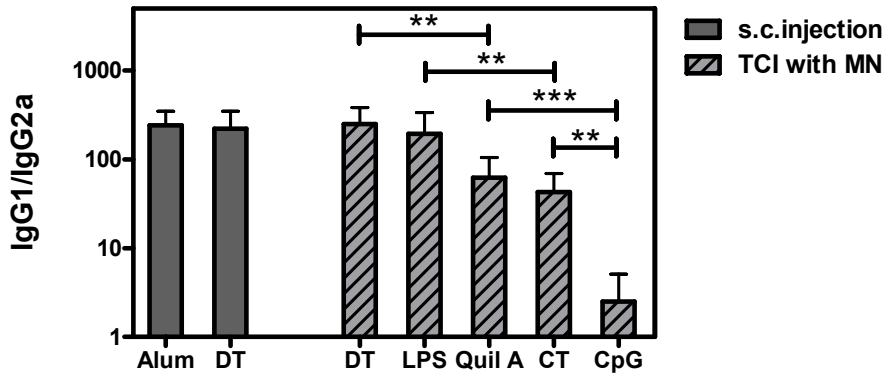

Figure 3. DT-specific IgG1/IgG2a ratios of individual mouse. Sera were collected on day 56, DT-specific IgG1 and IgG2a titers were measured with ELISA. IgG1/IgG2a ratios were calculated only using IgG2a responders. Data shown as mean + SD (n=8, ** $p<0.01$ and *** $p<0.001$, one-way ANOVA with Tukey posttest).

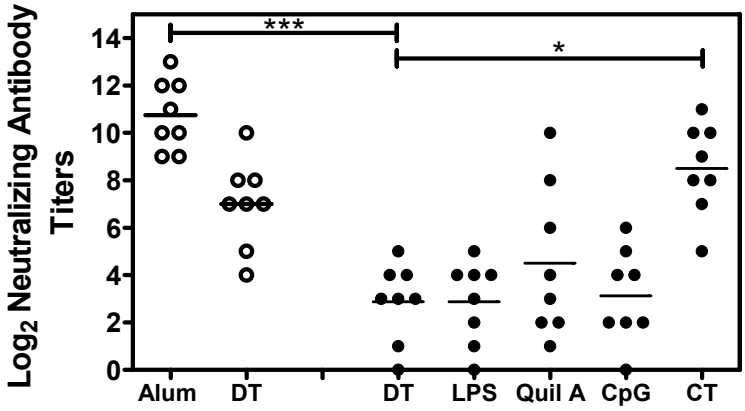

Figure 4. Diphtheria toxin-neutralizing antibody titers. Serum samples were collected after the 2nd boost (day 56) and determined with Vero cell test. Data are expressed as logarithm of the highest dilution that was still capable of protecting the Vero cells from the challenge of diphtheria toxin. (-●-: the TCI groups, **-○-:** the injection groups, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Kruskal-Wallis nonparametric test with Dunns posttest)

Protective immunity of DT in TCI with microneedle pretreatment

The protective immunity of DT with the serial of immune modulators was evaluated using the Vero cell test (Fig. 4). Although for LPS, Quil A and CpG groups the neutralizing antibody titers were not significantly different from those for plain DT, improvement was seen for the CT group ($p<0.05$). The mean titer was very close to that of the DT-alum injection group, and the difference was not significant ($p>0.05$). As expected, the groups that had received formulations on untreated skin (TCI) did not show detectable neutralizing antibody titers.

Discussion

This is the first study in which the immune modulatory activity of several known adjuvants was directly compared in TCI with microneedle array pretreatment, using DT as a model antigen. In this study, we first compared the immune responses of DT formulated with different adjuvants in TCI on intact or microneedle-pretreated skin. Then we focused our study on the modulation capability of these adjuvants to the immune responses of the latter.

Application of DT on intact skin did not induce a substantial immune response in the absence or presence of the tested adjuvants. The poor penetration of DT across the *stratum corneum* seems to be the limiting factor, since the

antibody titers of DT in TCI was strongly increased by microneedle array pretreatment. The skin barrier disruption and increased DT diffusion across the *stratum corneum* provided remarkable improvement to the immune response *via* TCI, similar as the tape-stripping method, however, through a practically easier and more controllable approach [26]. Co-administration of adjuvants was able to further augment the immunogenicity of DT and demonstrated marked differences between the immune stimulatory activities of the adjuvants. In the following section, the tested adjuvants are discussed individually concerning their mechanism of action and the role of the delivery route in the augmentation and modulation of DT immune response.

CT is a molecule with five nontoxic B subunits (CTB) surrounding a single, toxic A fragment (CTA). Both the CTB-mediated specific binding to the GM1 ganglioside receptors and the ADP-ribosyltransferase activity of CTA have been reported to be of importance for the immune stimulatory properties of CT [27, 28]. Whereas CT is a well-documented Th2 immune modulator in conventional and mucosal vaccination [28], our results point to a more Th1 biased response against DT when co-administered with CT on microneedle-pretreated skin. The Th1 skewing of CT in TCI was also reported by Stickings *et al.* using mutant diphtheria toxin, CRM197, as model antigen [29] and by Skountzou *et al*. using influenza vaccine [30]. This suggests that the Th1 skewing of CT is delivery-route dependent. Studies performed by Anjuere *et al.* using ovalbumin as prototype antigen clarified that CTB suppresses systemic Th1 responses when given by a mucosal route, but potentiates these responses when administered by TCI [31]. This finding underscores the role of the epithelial microenvironment in the regulation of immune responses and reflects the DC heterogeneity between mucosal tissue and skin.

The signaling of CpG starts by engagement of Toll-like receptor 9 (TLR9), followed by induction of pro-inflammatory cytokine (*e.g.* IL-12, TNF-α and IFN-γ) expression, and results in a Th1 biased response [32]. In our present study, CpG induced the highest IgG2a titers among all TCI groups and our results strongly support a skewing of the Th1/Th2 balance towards the Th1 direction, similar to the immune modulatory effect of CpG in mucosal and injection vaccination. Although at the site of application TLR9 is not constitutively expressed by the LCs, it is almost exclusively expressed by the keratinocytes in the upper and most differentiated layer of the epidermis. Their TLR9 expression and reactivity to CpG is reported to be greatly

up-regulated by transforming growth factor (TGF-α), a growth and differentiation factor present after skin barrier disruption and during wound healing [33].

Quil A is composed of immune stimulatory fractions extracted from the bark of the tree *Quillaja saponaria*. The Quil A concentration (1.43 mg/ml) used in the applied formulation was well above its reported critical micelle concentration of 0.3 mg/ml [34]. The average particle size of the DT-Quil A dispersion in PBS was about 56 nm with a PDI of 0.10, as measured by dynamic light scattering. Quil A is a potent Th1 biased adjuvant when given by i.m. injection [35]. In the current study, it improved the IgG titers marginally as compared to the non-adjuvanted DT ($p<0.05$ after the 1st boost; $p>0.05$ after the 2nd boost). The ratios of anti-DT IgG1/IgG2a titers were significantly lowered as compared to plain DT ($p<0.01$), suggesting that it skewed the response towards the Th1 direction.

LPS, major outer membrane constituent of Gram-negative bacteria, stimulates APCs through TLR4, induces high levels of pro-inflammatory cytokines and usually leads to a strong Th1 response [36]. The DT- *lpxL1* LPS dispersion applied in our study had a bigger average particle size than Quil A, about 300 nm with a PDI of 0.45, indicating that relatively large complexes had been formed. The effect of the *lpxL1* LPS mutant on the anti-DT IgG, IgG1, IgG2a and the neutralizing antibody titers was not significant ($p>0.05$). This may be due to the following factors: i) LCs do not express TLR4 and do not respond to bacteria or LPS [37]. TLR4 expression by mouse keratinocytes also seems limited. Besides few data available from mice, reports on TLR4 expression by human keratinocytes are conflicting [38, 39]; ii) Although little is known about the actual diffusion of these dispersions across the conduits induced by the microneedles, previous studies of our group showed that fluorescently labeled particles of ca. 200 nm did pass through the conduits created by the same microneedle arrays as used in the present study [18]. However, as no quantitative information is available, it is possible that only a limited amount of the DT-*lpxL1* LPS dispersion reached the DCs in the dermis; iii) the LPS mutant may work in a less efficient way as the original LPS in the epidermal/dermal microenvironment. Further studies are required to elucidate the exact mechanism. The different reactivity of skin immune system to CpG and LPS may be involved in a strategic control of host defense to the bacterial commensal skin flora [37].

Alum did not show any adjuvanticity when applied on microneedle-pretreated skin. It induced lower IgG and IgG1 titers than those of non-adjuvanted DT group after each vaccination. Furthermore, alum did not induce detectable IgG2a titers and provided no protection in the Vero cell test after the 2nd boost (data not shown). This may partially be due to the size of DT-alum particles, several microns in diameter, which prevents their diffusion through the conduits in sufficient amount to exert a 'depot' effect [40]. The adjuvanticity of alum could also be dependent on the epithelial microenvironment; the danger signal it induces through injection, the uric acid production [41], may not be provoked in the dermis and epidermis.

Interestingly, when comparing IgG subtype titers and neutralizing antibody titers between CpG and Quil A groups (Fig. 1 and Fig. 4), the CpG group, with similar IgG titers, but much higher IgG2a titers than the other, induced similar to slightly lower neutralizing antibody titers, which indicates that IgG2a might not contribute to toxin-neutralization. This was confirmed by the comparison between CpG and CT groups, also showing that higher IgG2a titers did not result in higher neutralizing antibody titers. IgG1 seems to be the main neutralizing antibody for protection, as IgG1 titers significantly correlated with neutralizing titers. However, IgG1 titers could not be used to predict individual neutralizing antibody titers with great accuracy, which is in line with a diphtheria vaccination study done in human infants [42]. Therefore, from an application perspective, CpG is more suitable for anti-viral immune responses such as influenza vaccination where IgG2a provides the main immune protection and a Th1 biased response is more desired [43].

In this study, a high dose of DT, 20 times of that used in the s.c. injection group, was applied onto the skin. It is the same as the dose of DT used by Glenn *et al.* in previous studies [26, 48]. High doses were used to ensure sufficient delivery and high antigen concentration. In fact, only a fraction of DT applied actually enters epidermis/dermis. A limiting factor for diffusion of the antigen into the skin is that the mice used for this study can only be kept under anesthesia for about one hour. This is not a limitation of the transcutaneous route, but a limitation of the animal model used. The high doses used until now also indicate that formulation improvement to increase the efficiency of vaccination is necessary. Conjugation and encapsulation of adjuvants into vaccine-containing particles and specific DC and LC targeting approaches could be attractive strategies to further improve the potency of vaccine formulations for TCI.

Conclusion

We have shown that the type of adjuvant used has a significant effect on the immune response and protective immunity against DT in TCI. The epithelial microenvironment and DC heterogeneity also play an important role in the regulation of the immune response. This delivery method is applicable to many other vaccines by formulating with proper adjuvants and holds a lot of promise for future use.

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Chapter 5

Preparation and characterization of diphtheria toxoid-loaded elastic vesicles for transcutaneous immunization

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Introduction

Although vaccination *via* the needle has led to tremendous advances in the control of many infectious diseases [1], there is an increasing demand for non-invasive vaccine delivery. Pediatric vaccine program is the best example where a high number of antigens need to be introduced in a short period of time, leading to complex antigen mixtures and many injections. One of the promising routes for non-invasive vaccine delivery is the transcutaneous route, as the skin is a highly immune active organ and easily accessible. Human skin is composed of three layers, from the surface down, the *stratum corneum*, the epidermis and the dermis. The epidermis has a dense population of bone marrow-derived dendritic cells, referred to as Langerhans cells. These Langerhans cells, which travel to the draining lymph nodes after activation, are highly efficient antigen presenting cells [2, 3]. They are the targets of transcutaneously delivered vaccines.

A major hurdle for successful transcutaneous immunization (TCI) is the barrier function of the skin, which resides in the uppermost layer, the *stratum corneum*. This layer is composed of dead, cornified, tightly packed keratinocytes embedded in lipid lamellar regions. This layer is considered to be only minimally permeable for high-molecular-weight substances, such as antigens [4], while chemical penetration enhancers and also physical means, such as iontophoresis, only have shown success in delivering small molecules across the skin barrier [5]. Therefore, the greatest challenge in TCI is improving the transport of antigens across the *stratum corneum*. Recently, several studies have reported that the use of antigen-containing occlusive patches induced strong immune responses in an animal model and human volunteers. [6-8]. However, successful vaccination largely depends on the antigen type and very high antigen doses have been used in these occlusive patch studies. Therefore, there is still an urgent demand to improve the delivery of antigens into the skin.

One promising approach for transcutaneous delivery of antigens is the use of vesicular formulations, especially elastic vesicles that have highly deformable bilayers [9, 10]. For instance, hepatitis B surface antigen (HBsAg)-loaded elastic liposomes applied onto intact mouse skin elicited robust systemic and mucosal antibody responses against HBsAg [11]. Transfersomes® composed of soybean phosphatidylcholine, sodium cholate and sodium dodecyl sulfate have also been used in TCI studies. Successes were achieved with these

vesicles in combination with antigens such as human serum albumin [12], gap junction proteins [13] and tetanus toxoid [14] in murine models. Depending on the antigen type, dose, immunization schedule, presence of co-stimulatory factors and vesicle composition, immunization with antigen formulations based on elastic vesicles can induce effective immune responses with serum IgG levels comparable to those obtained after subcutaneous injection.

A new generation of elastic vesicles were introduced 10 years ago [15], consisting of the bilayer-forming surfactant L-595 (sucrose-laurate ester) and the micelle-forming surfactant PEG-8-L (octaoxyethylene-laurate ester). These elastic vesicles were able to penetrate pores much smaller than their diameter [16]. Recently, it was suggested that these elastic vesicles may act as carrier systems; after non-occlusive application elastic vesicles and bound fluorescent label followed the same route of penetration into the *stratum corneum* and intact vesicle structures have been visualized in the *stratum corneum* [17]. Furthermore, transport studies of small drugs such as pergolide, lidocaine, ketorolac and rotigotine [18-21] demonstrated that higher transport rates of these drugs, compared to the same drug in buffer solutions or with rigid vesicles, can only be achieved when drug molecules are physically associated with the elastic vesicles. The drug-loaded elastic vesicles were applied non-occlusively since the trans-epidermal osmotic gradient is believed to be the driving force for the transport [22]. As these vesicles act as drug carrier systems for low-molecular-weight drugs, they may hold promise as antigen delivery systems by enhancing antigen partitioning and transporting to the deeper layers of the *stratum corneum*. If intact antigen-loaded vesicles are in the *stratum corneum* close to the viable epidermis, the antigen might be released and diffuse into the viable epidermis and, after reaching the Langerhans cells, trigger an immune response.

The aim of the current study was to develop DT-containing elastic vesicle formulations for TCI. DT was chosen as a model antigen, since it has been used in murine models for TCI studies in combination with mucosal adjuvants [23, 24], physical methods such as electroporation [25] and local hyperthermia [26]. However, DT alone cannot induce protective immunity when applied on intact mouse skin because of the low intrinsic immunogenicity of plain DT and/or the insufficient amount of DT delivered *via* the skin [23]. One approach to improve the delivery of DT through the skin and make it more immunogenic is utilizing carrier systems such as elastic vesicles. As elastic vesicles act as carrier, physical association of DT with the

vesicles is expected to be crucially important. Therefore, in the current study, the vesicle composition, DT concentration, pH and buffers were optimized to obtain high DT association levels as well as good colloidal stability of the DT-vesicle formulations.

Materials and methods

Materials

Diphtheria toxoid (MW: 58 kDa; isoelectric point about 4.7), horse anti-DT and horse radish peroxidase conjugated anti-DT were provided by the NVI (Netherlands Vaccine Institute, Bilthoven, The Netherlands). Sucrose-laurate ester (L-595; 30% mono-, 40% di-, and 30% triesters, mean MW 734) was kindly supplied by Mitsubishi Kasei (Tokyo, Japan). Octaoxyethylene-laurate ester (PEG-8-L; mean MW 552) was a gift from Lipo Chemicals (Paterson, New Jersey, US) and sodium bistridecyl sulfo succinate (TR-70; mean MW 585) was a gift from Cytec B.V. (Rotterdam, The Netherlands). Tween 80 was purchased from Merck (Darmstadt, Germany). Tween 20, lyophilized bovine serum albumin (BSA) and Folin Ciocalteu's phenol reagent were obtained from Sigma (Zwijndrecht, The Netherlands). Chromogen 3, 3', 5, 5'-tetra-methylbenzidine (TMB) and the substrate buffer were purchased from Biosource B.V (Etten-Leur, The Netherlands). All other chemicals used were of analytical grade and all solutions were prepared with distilled water.

Methods

Preparation of the vesicle dispersion and DT-loaded vesicle formulations

Elastic vesicles used in this study consisted of the bilayer-forming surfactant L-595, the micelle-forming surfactant PEG-8-L, and the stabilizer TR-70 in the molar ratio of either 5:5:1 or 7:3:1. The 5:5:1 and 7:3:1 ratios were selected as in previous studies these ratios resulted in stable dispersions containing medium elastic (7:3:1) or very elastic (5:5:1) vesicles [15-17]. For clarity, all vesicle compositions will be indicated below only by the molar ratio between the two main components L-595 and PEG-8-L. Vesicles were prepared using a modified sonication method [10]. In brief, the surfactants were dissolved in ethanol, while TR-70 was dissolved in an ethanol/isopropanol mixture. The solutions were mixed in an appropriate ratio. The organic solvents were then evaporated overnight in a vacuum centrifuge and the remaining surfactants were dispersed in 0.01 M acetate buffer (Ac, sodium acetate buffer, 8.4 mM

$H_2C_2O_4$ and 1.6 mM $Na_2C_2O_4$, pH 4.0), citrate buffer (CB, 3.2 mM $H_3C_6H_5O_7$ and 6.8 mM $Na_3C_6H_5O_7$ adjusted by adding 0.1 M HCl, pH 5.0) or phosphate buffer (PB, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.4). After adding DT, the final concentration of total surfactant was 10% w/w. DT with the same buffer was added to the vesicle dispersion to a final concentration of 1.7 mg/ml. The selected concentration of DT derives from the dose and volume of formulation needed in future *in vivo* studies. Subsequently, vesicle dispersions were sonicated for 3×5 s using a Branson Sonifier 250 (Branson Ultrasonics, Danbury, UK) with 3 mm micro tip at 70 mW energy output. DT has lest solubility in buffer at pH 4.7 due to its isoelectric point (pI, unpublished data). It was found that solubility of DT in CB is below 1.7 mg/ml at pH 4.5 (visible precipitation) and above 1.7 mg/ml at pH 5.0. Therefore, to obtain a DT-containing vesicle formulation at pH 4.5, the surfactants mixture after vacuum centrifuge was first rehydrated in CB at pH 5.0. After adding DT and sonication, a small amount of 0.1 M HCl was added to the DT-vesicle suspension, lowered the pH to 4.5. This resulted in a stable DT-containing vesicular formulation. The final DT concentration was kept constant for all the formulations. Stability studies were carried out from day 1 till day 14 after preparation. During these studies the formulations were stored at 4 ºC.

To investigate whether electrostatic interactions play a role in DT-vesicle association, DT formulations were also prepared with 0.01 M phosphate buffered saline at pH 7.4 (PBS, PB with 153 mM NaCl) and 0.01 M citrate buffered saline at pH 5.0 and 4.5 (CBS, CB with 153 mM NaCl). To achieve vesicles with similar size, the sonication period was extended to 80-100 s at this higher salt concentration. The formulations were stored at 4 ºC and the association studies were carried out within 3 days after preparation.

Particle size and ζ-potential measurements

The vesicle size and polydispersity index (PDI) of all formulations were measured by dynamic light scattering (DLS) using a Malvern Zetasizer 3000 HSA (Malvern Ltd., Malvern, United Kingdom). All size measurements were performed at 25 °C at an angle of 90° between laser and detector. Before measuring, all samples were diluted with their original buffer. For estimation of the surface charge of the DT-associated vesicles, 3.0 µl of vesicle formulation were diluted in 1.00 ml of 1 mM CB or PB (pH corresponding to that of the formulation) and the ζ-potential was measured by laser Doppler electrophoresis using the same device. Formulations were measured at

regular time-intervals during a period of 2 weeks after preparation to check the colloidal stability.

Size exclusion chromatography (SEC)

To determine the amount of DT associated with vesicles in the formulations, SEC was performed with a Sepharose CL-4B (Amersham, Uppsala, Sweden) column (30 cm long, cross-sectional area 0.79 cm^2). A dual λ absorbance detector (Waters 2487 EMI ISM Instrument, Etten-Leur, The Netherlands) set at 280 nm was used to monitor the separation of the free DT and vesicles-associated DT fractions. Empty vesicles and free DT in buffer solution were used to determine the collecting time windows of these fractions. For each formulation, the corresponding formulation buffer was used as eluent at a flow speed of 0.44 ml/min.

Protein and antigenicity assay

The protein recovery rate of the DT from the fractions collected after SEC were determined by a modified Lowry-Peterson protein assay [27]. The antigenicity of the DT from SEC fractions (relative to that of untreated DT) was measured by a sandwich ELISA, using horse anti-diphtheria serum and a horse anti-diphtheria peroxidase conjugate [28]. ELISA was also performed with sonicated DT, DT-vesicle formulations at pH 4.5 and pH 5.0 to study the preservation of DT antigenicity. Data were analyzed by use of the principles of parallel-line analysis of the OD-log concentration plots as described by Tierney *et al*. [29].

Fluorescence spectroscopy

DT-vesicle formulations and DT solutions before and after sonication were studied by fluorescence spectroscopy as described elsewhere [30]. In brief, DT incubated at 4 ºC for 4 h with 4 M guanidine-HCl (denatured DT) served as control [28, 31]. The selected excitation wavelength was 295 nm (band width 2.5 nm) and the emission spectra of the toxoid samples (140 µg/ml) were recorded from 300-450 nm (band width 5 nm) at 25 ºC with a fluorescence spectrometer (Perkin Elmer LS50B, Massachusetts, US). For each sample the emission spectrum was determined from five averaged scans (corrected for background fluorescence).

SDS-PAGE

DT solutions before and after sonication and DT-vesicle formulations were diluted with corresponding buffer to a concentration of 0.3 mg/ml. Non-reducing loading buffer (4x) was added to the samples. SDS-PAGE molecular weight markers (broad range; Bio-Rad, Veenendaal, The Netherlands) were used for calibration. Approximately 3 μg protein was loaded on the gel (4-20% precise protein gel, Pierce, Etten-Leur, The Netherlands) and run at 100 V. Protein bands were visualized by Coomassie brilliant blue.

Results

Effect of DT-vesicle composition on particle size

In our first series of studies the stability of the selected vesicle formulation varying in DT concentrations between 0.2 and 1.7 mg/ml DT was examined by visual inspection and DLS. The pH of the formulations was varied between 4.0 and 7.4. When the formulations were prepared at pH 4.0, pH 4.5, 5.0 and 7.4 with a DT concentration of 0.2 mg/ml, visible precipitation was observed for the 7:3 vesicles, whereas the 5:5 vesicle formulations with up to 1.7 mg/ml DT remained stable at pH values of 4.5, 5.0 and 7.4 (Table I). Using DLS, a z-average diameter of 80-110 nm was measured for the 5:5 vesicles. Therefore in the presence of DT the 5:5 vesicles appeared to be more stable than the 7:3 vesicles. The 5:5 vesicle formulations were selected for more detailed characterization at pH 4.5, 5.0 and 7.4.

Colloidal stability of 5:5 DT-loaded vesicles

The stability of the 5:5 vesicles was evaluated by measuring the particle size of DT-loaded (1.7 mg/ml DT) and empty vesicles by DLS (Table I). The z-average mean diameter of the vesicles ranged from 80 to 110 nm. The PDI of chosen formulations was less than 0.3, which indicates a moderately homogenous size distribution. The vesicles were colloidally stable at 4 ºC during a period of at least 14 days after preparation. Interestingly, the size of DT-loaded vesicles was marginally larger than that of the empty vesicles at pH 4.5, while the DT-loaded and empty vesicles at pH 5.0 and pH 7.4 did not differ in size.

Table I. Characteristics of DT-vesicle formulations in buffers with different pHs. Data represent mean ± SD of three batches.

L595:PEG-8-L:TR-70 = 5:5:1 (Molar ratio)						
pH	Buffer**	[DT] mg/ml	Size (nm)*			ζ-potential at day 1 (mV)
			Day 1	Day 7	Day 14	
7.4	PB	0	96.1 ± 2.8	92.2 ± 4.0	91.9 ± 2.9	-73.7 ± 3.4
7.4	PB	1.7	96.9 ± 2.1	95.8 ± 1.0	96.5 ± 2.3	-75.9 ± 3.6
5.0	CB	0	84.1 ± 2.4	85.2 ± 2.1	84.4 ± 2.5	-86.9 ± 3.4
5.0	CB	1.7	79.7 ± 2.1	80.3 ± 2.7	79.6 ± 2.9	-83.3 ± 3.5
4.5	CB	0	84.5 ± 4.9	85.1 ± 5.1	84.3 ± 4.4	-84.2 ± 1.4
4.5	CB	1.7	103.5 ± 7.2***	118.3 ± 14.9	114.7 ± 5.0	-77.7 ± 3.6***
4.0	Ac	0	80.2 ± 4.4	N.P.**	N.P.	N.P.
4.0	Ac	0.2	88.8 ± 5.7	-**	-	-

*The PDI ranged from 0.2 to 0.3.

**N.P.: Not performed; -: Visible precipitation.

***: Significantly different from the corresponding formulation without DT (p<0.05).

As the isoelectric point of DT is about 4.7, a pH shift from 5.0 to 4.5 will change the net charge of DT molecules from negative to slightly positive, which may affect the interaction between DT and the vesicles. Therefore, the surface charge of DT-loaded vesicles was measured using laser Doppler electrophoresis (Table I). The ζ potential of the vesicles varied between -74 mV (vesicles at pH 7.4) and -87 mV (vesicles at pH 5.0). The DT-loaded vesicles prepared at pH 4.5 have a less negative surface potential than the vesicles without DT. This difference in surface potential is probably due to the presence of association between DT and vesicles. At pH 5.0, no difference in ζ potential was observed for vesicles prepared in the presence and absence of DT, indicating that less DT is associated to the vesicles at this pH. The association between DT and vesicles as a function of pH and ionic strength was examined in more details.

Association of DT with 5:5 vesicles

pH-dependence (pH selection)

In these studies, SEC in combination with Lowry-Peterson and ELISA assays were used to obtain information about the DT-vesicle association in the formulations. The pH of the DT-vesicle formulations was 4.5, 5.0 in CB and 7.4 in PB, and the DT concentration was 1.7 mg/ml. DT's association to the vesicles exceeded 70% at pH 4.5 and was below 20% at pH 5.0 and 7.4 (Fig.

1a). The recoveries measured by Lowry-Peterson and ELISA assays were very similar (Fig. 1a and 1b), indicating that associated DT had maintained its antigenicity. At pH 4.5 almost 80% of the protein was measured to be associated with the vesicles by ELISA.

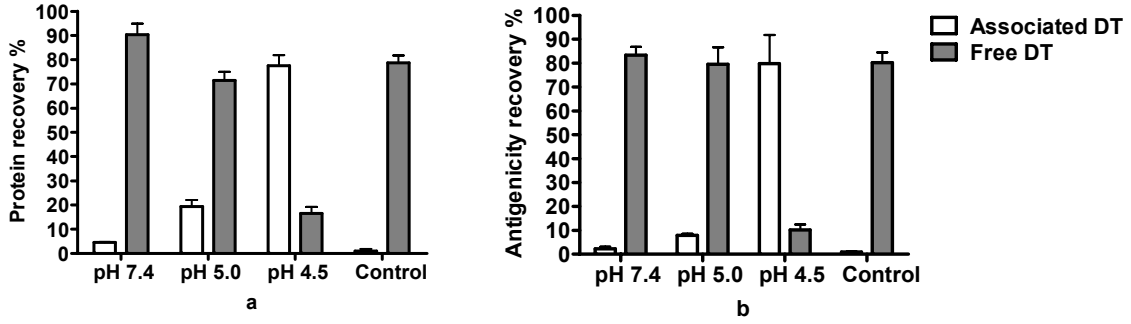

Figure 1. Effect of pH on association of DT and vesicles (5:5). Protein recovery (a) and antigenicity recovery (b) were determined after SEC fractionation of DT-vesicles at pH 4.5, 5.0 and 7.4. DT solution (1.7 mg/ml) in citrate buffer at pH 5.0 was used as control. Data shown are mean + SD (n=3).

Ionic strength (buffer selection)

Using SEC and subsequently the Lowry-Peterson protein assay, it was shown that the association of DT with the vesicles was independent on the ionic strength at all pH values (Fig. 2). This indicates that the ionic strength has little influence on the DT-vesicle association. Considering the shorter sonication time required for the preparation procedure and the low salt concentration leading to less remainder when applying the formulation non-occlusively onto the skin, DT-vesicles prepared in CB at pH 4.5 were chosen for further studies.

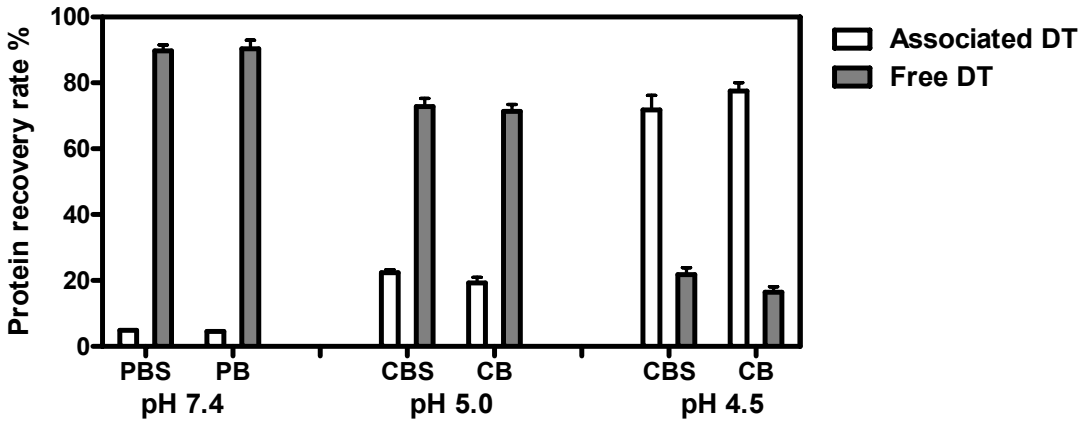

Figure 2. Influence of ionic strength on the DT-vesicle association (5:5 vesicles with 1.7 mg/ml DT). Protein recovery as detected by Lowry-Peterson protein assay was compared between PB and PBS, between CB and CBS at pH 5.0 and 4.5. Data shown are mean + SD (n=3).

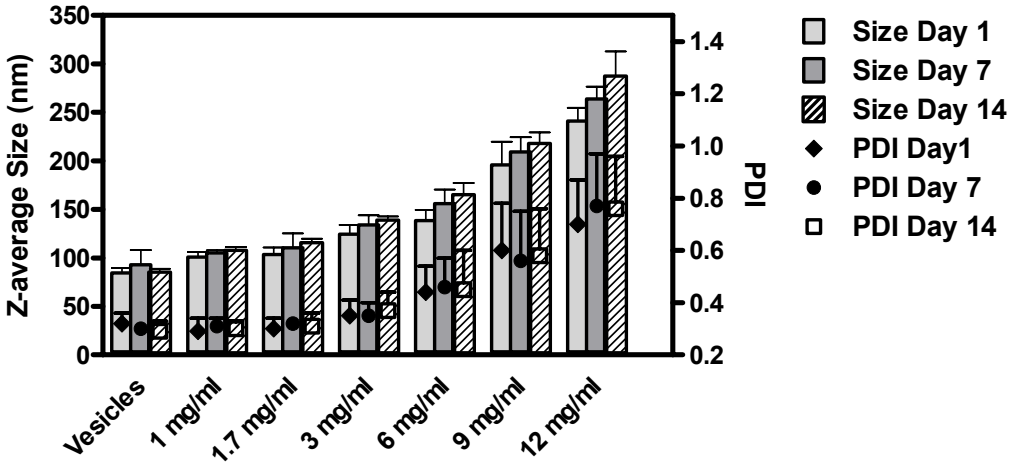

Figure 3. Effect of DT concentration on the particle size and PDI of DT-loaded vesicles (5:5) prepared at pH 4.5. Data represent mean + SD of three batches.

Loading capacity (DT concentration selection)

To study the loading capacity and stability of the 5:5 DT-loaded vesicles at pH 4.5, formulations with DT concentrations varying from 1.0 to 12.0 mg/ml in CB were prepared and evaluated for short-term colloidal stability during 2 weeks. The initial particle size increased with higher PDI at increasing DT concentrations (Fig. 3).The particle size tended to increase during storage, especially for the formulations containing high DT concentrations (Fig. 3). The ζ potential of DT-loaded vesicles (Fig. 4) became gradually less negative with increasing DT concentration, probably due to more DT association to the vesicles. To verify the ζ potential results quantitatively, the association of DT as a function of DT concentration was studied by SEC in combination with the Lowry-Peterson protein assay and ELISA. Formulation with 1 to 9 mg/ml of DT presented 75-95% association to the vesicles (Fig. 5). Similar values were seen for DT concentrations up to 3 mg/ml when the antigenicity was determined using ELISA (Fig. 5). However, formulations with 6 and 9 mg/ml DT showed a significantly lower antigenicity recovery than the detected protein recovery.

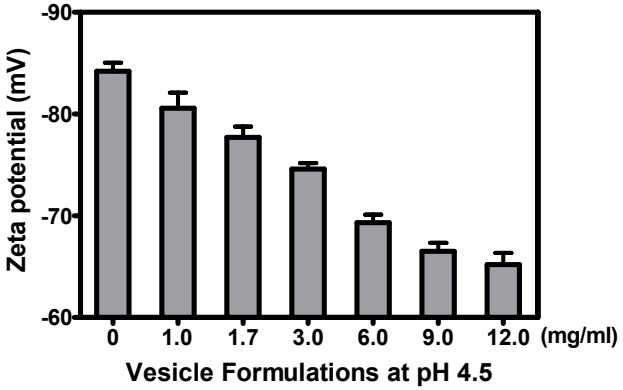

Figure 4. Z potential of empty vesicles and DT-loaded vesicles (5:5) with DT concentration from 0 to 12 mg/ml. Data represent mean + SD of three batches.

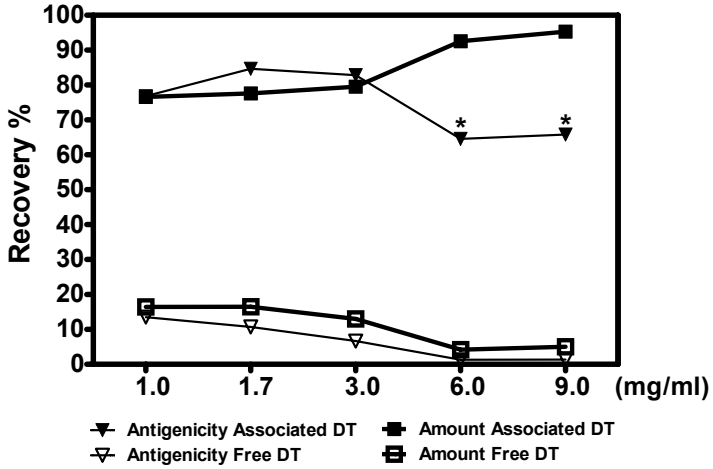

Figure 5. Effect of DT concentration (1 to 9 mg/ml) on the association of DT with vesicles (5:5) at pH 4.5. Protein recovery and antigenicity recovery were determined after SEC fractionation of the DT-vesicles. Data shown are mean of three batches. *indicates that difference between protein recovery and antigenicity recovery at DT concentration of 6 and 9 mg/ml are significant (p<0.01).

Effect of sonication and low pH on the DT structure

From the above studies, stable DT-vesicle formulations were obtained in CB at pH 4.5 by sonication. Since DT molecules may be sensitive to environmental factors such as sonication, low pH and the presence of vesicles; ELISA, fluorescence spectroscopy and SDS-PAGE were performed to determine whether the DT structure was preserved during formulation.

DT-vesicle formulations at pH 4.5 and pH 5.0, DT solution in CB at pH 5.0 after 3×30 s sonication were measured with ELISA and compared to the control, no significant difference was observed. The conformation of DT was studied by intrinsic tryptophan fluorescence spectroscopy. Consistent with

previous findings [30], untreated DT showed an average maximum fluorescence emission at a wavelength of 335 nm, whereas the maximum fluorescence emission wavelength of DT denatured by guanidinium hydrochloride was shifted to 353 nm, caused by an increased exposure of the tryptophan residues to the aqueous surroundings. The maximal emission wavelength of the DT-vesicle formulation at pH 4.5 and pH 5.0 and the DT solutions after sonication (3×30 s) remained at 335 nm, which indicates the absence of conformational changes of DT.

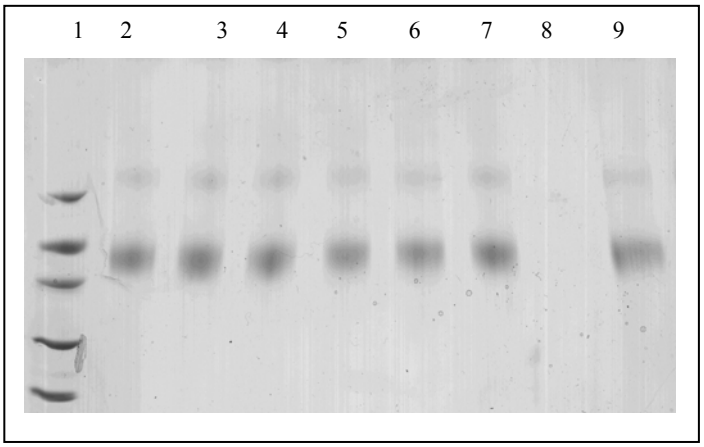

Figure 6. SDS-PAGE of DT solutions before and after sonication and DT-vesicle formulations. From lane1 to 9 are: 1) Broad range MW Marker; 2) DT in PBS as control; 3) DT in PBS after 3×10 s sonication; 4) DT in PBS after 3×30 s sonication; 5) DT vesicles in CB at pH 5.0 (3×5 s sonication); 6) DT vesicles in CBS at pH 5.0 (80 s sonication); 7) DT vesicles in CB at pH 4.5 (3×5 s sonication); 8) Empty vesicle control; 9) DT vesicles in CBS at pH 4.5 (80 s sonication).

SDS-PAGE under non-reducing conditions of DT solutions after 3×10 s or 3×30 s sonication, the DT-vesicle formulation in CB or CBS at pH 4.5 and pH 5.0 showed identical bands of similar intensities at the same positions as the untreated DT (Fig. 6). This indicates that the sonication used for formulation preparation and DT association with vesicles did not induce covalent aggregation or fragmentation of the protein.

The combined results from SDS-PAGE, fluorescence spectroscopy and ELISA point to full preservation of the DT structure after its formulation with 5:5 vesicles in CB at pH 4.5.

Discussion

In several TCI studies, DT and also other antigens derived from bacterial ADP-ribosylating exotoxins have been investigated. However, typical doses in these studies were 100 µg per vaccination [32, 33], which is much higher than

doses for injection. Therefore there is an urgent need for more efficient dermal vaccine delivery systems allowing lower antigen doses. In previous studies several groups report successful immunization *via* the dermal route using elastic vesicles [11-13]. It was also reported that surfactant-based vesicles served as adjuvants for co-administrated antigens given by injection [34] and topical immunization [35]. In the study described in this paper, the main focus was to formulate DT in colloidally stable elastic vesicle dispersions to be used for TCI.

Selection of DT-vesicle formulations

The characteristics of DT-vesicle formulations were strongly dependent on the vesicles composition and pH. As it has been reported that vesicles with L-595/PEG-8-L molar ratios of 7:3 and 5:5 are both elastic [16], these surfactant ratios were chosen to prepare DT-loaded vesicles. Our present studies revealed that the 5:5 vesicles are more stable than the 7:3 vesicles in the presence of DT. As oxyethylene headgroups are known to promote entropic stabilization [36], the higher levels of PEG-8-L in the 5:5 vesicles may count for the higher stability as compared to the 7:3 vesicles.

Elastic vesicles may act at least partly as a carrier system. Therefore, it is crucial to associate DT with the vesicles. At pH 4.5 DT is slightly positively charged and not readily dissolved in the buffer solution. Therefore at this pH DT-membrane interactions are facilitated, resulting in more than 70% of DT associated with vesicles. It has been reported that DT undergoes irreversible conformational change at pH values lower than 4.2, which facilitates the interaction between DT and lipid membrane [31]. However, in our studies the DT vesicle formulations at pH 4.0 were not stable. Therefore the formulations at a pH lower than 4.5 were not further investigated.

For vaccination, non-occlusive application and drying of the formulation during the application period (typically 1 h) is important for the unique interactions between elastic vesicles and skin [21, 22], and only a small volume of vesicle formulations can be applied on the limited area of mouse skin (~30 µl/cm^2). For dermal delivery, antigen concentration is normally of more importance than dose. Therefore, in order to determine the highest DT concentration at which stable vesicle formulations could be prepared, vesicle formulations with a DT concentration ranging from 1 to 12 mg/ml were examined. The results indicate that vesicle dispersions at DT concentrations higher than 3 mg/ml were not optimal for several reasons, namely; i) at DT

concentrations of 6 mg/ml and higher, the PDI increased rapidly indicating less homogeneous dispersions; and ii) association studies showed that at DT concentrations up to 3 mg/ml, protein recovery and antigenicity follow the same trend, whereas at DT concentrations of 6 mg/ml and higher, the recovered antigenicity was significantly lower than the amount of protein associated with vesicles. Considering the high PDI of these formulations and the visible aggregates in some of the 12 mg/ml DT-vesicle formulations, the fraction of vesicle-associated DT from 6 and 9 mg/ml DT formulations might contain DT aggregates as well, which might reduce the accessibility of the detecting antibodies to the epitopes of DT molecules. In conclusion, vesicle dispersions at pH 4.5 and a DT concentration up to 3 mg/ml appear to be optimal for TCI.

Mechanism of association

A pH-sensitive interaction between DT and vesicles was found in the current study, which is consistent with previous studies [31]. During SEC separation, it is difficult to explain the relatively low (~80%) recovery rate of DT in CB at pH 5.0 from the column (Fig. 1). However, nearly 100% of DT was recovered from SEC in the presence of the vesicles, probably due to the solubilizing properties of vesicle components. In the SEC washout curve, a tail part following the peak of vesicles was observed, indicating the possible presence of micelles formed mainly by PEG-8-L. Since DT has a pI of about 4.7, at pH 5.0 and 7.4, DT and vesicles are both negatively charged. Under these conditions DT remained ionized in solution and hardly any DT was associated with the vesicles. In contrast, at pH 4.5 free DT tends to form aggregates, possibly due to a decrease of the net charge and increase of hydrophobicity, whereas stable DT-vesicle formulations could be obtained. This indicates that DT is stabilized by association with the vesicles. As the ionic strength of the buffer did not affect the DT-vesicle association, hydrophobic interactions may contribute largely to the association between DT and vesicles.

However, the reduction in surface potential of DT-loaded vesicles compared to the empty vesicles at pH 4.5 indicates the interaction between slightly positively charged DT and the negatively charged vesicles. Therefore, the DT-vesicle electrostatic interactions might also play a role. Probably the opposite charge of the DT and the vesicles triggers the initial (long-range) electro statically driven force. Once associated, the hydrophobic interactions may contribute significantly because of the short-range van der Waals forces.

Therefore probably the hydrophobic domain of DT is intercalated in the bilayer of the vesicles. Furthermore, when applying the DT-loaded vesicle fraction onto the SEC column again, only vesicle-associated DT but no free DT could be eluted at pH 4.5, indicating a stable interaction between DT and the vesicles (data not shown). The order of DT addition to the formulation (before or after sonication) did not affect the DT-vesicle association in CB at pH 4.5 and pH 5.0 (data not shown), indicating that energy supply into the system by sonication did not promote association or cause degradation but only served to reduce the particle size of the vesicles.

Conclusion

In the present study, stable DT-containing vesicles with a high loading capacity were developed as a potential candidate for transcutaneous immunization studies aiming at more efficient vaccination.

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Chapter 6

Transcutaneous immunization studies in mice using diphtheria toxoid-loaded vesicle formulations and a microneedle array

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Introduction

Vaccination is a cost-effective way to fight infectious diseases. Currently most vaccines are administered by subcutaneous (s.c.) or intramuscular injection. The problems with administration *via* these routes are that they are painful, cause stress (especially in children), require trained personnel and may lead to side effects. This results in incomplete vaccination coverage and, hence, unnecessary deaths from vaccine-preventable diseases. Consequently, research is focused on alternative vaccine delivery through oral [1], dermal [2], nasal [3] and pulmonary [4] routes. Vaccination *via* the dermal route is of particular interest since the skin is the biggest organ and is easily accessible. Antigen-presenting cells (APCs) in the skin, in particular, the Langerhans cells (LCs) in the epidermis and the dermal dendritic cells (dDCs) in the dermis are the targets for transcutaneous immunization (TCI), *i.e.* topical vaccine application onto intact or pretreated skin. Epidermal LCs usually represent about 1% of the total epidermal cell population whereas covering 20% of the skin surface [5]. Upon contact with certain antigens together with danger signals, *e.g.* skin barrier disruption or inflammation, LCs and dDCs become activated. They capture antigens/pathogens, migrate to the peripheral draining lymph nodes, process and present the antigen to the naïve T cells and initiate immune responses [6]. However, a major hurdle is to overcome the *stratum corneum*: the upper-most layer of the skin which acts as a barrier. This layer consists of corneocytes embedded in a highly organized crystalline lamellar structure of the intercellular lipid matrix. The unique arrangement of this layer (15-20 μm thick in humans and thinner in mice) makes it impermeable for macromolecules, including antigens [7].

In the 1970s, Gerstel and Place proposed the use of microneedle arrays as a way to overcome this barrier in a minimally-invasive manner[8]. However, it was not employed in (trans)dermal drug delivery studies until about ten years ago when the technology for fabrication in micron dimensions became available and cost-effective [9]. After the skin is treated with a microneedle array, tiny conduits are formed transiently with little pain sensation. Antigens are able to diffuse through the conduits to the APCs in the epidermis and dermis [10]. It was shown in Chapter 3 that microneedle array-pretreatment leads to major improvement in the immunogenicity of topically applied diphtheria toxoid (DT) in mice. Other studies have also demonstrated the promise of microneedle array-aided TCI: Matriano *et al*. studied the dosing of a model antigen, ovalbumin, and the kinetics and the magnitudes of antibody

titers induced in microneedle-mediated TCI in hairless guinea pigs [11]; Widera *et al*. investigated the influences of important fabrication parameters, *e.g.* the needle length, needle density, and covered skin area of the ovalbumin-coated microneedle arrays, on the effectiveness of TCI [12].

Another approach to overcome the skin barrier in TCI is to formulate antigens with vesicles. Elastic vesicles, consisting of highly deformable bilayers, have been reported to be able to improve transdermal drug delivery. These vesicle formulations were applied non-occlusively as the transdermal osmotic gradient is considered the driving force of diffusion across the *stratum corneum* [13]. For example, Transfersomes® have been used successfully for a range of small molecule drugs, including steroids, non-steroidal anti-inflammatory drugs and local anesthetics [14-16], as well as peptides and proteins, *e.g.* cyclosporin A and insulin [17, 18]. Moreover, TCI with hepatitis B surface antigen-loaded elastic liposomes and tetanus toxoid-loaded Transfersomes® has been shown to elicit immune responses equivalent to those produced by intramuscular injection of alum-adsorbed antigens in mouse and rat, respectively [19, 20]. In our group, elastic vesicles composed of surfactants have previously been developed. After non-occlusive application on the skin, vesicle structures were observed in the deeper layers of the *stratum corneum* close to the *stratum corneum*-viable epidermis junction. Enhanced delivery of lidocaine, rotigotine and ketorolac was observed when the drug was associated with vesicles and the formulation applied on the skin in a non-occlusive manner [21-23]. Therefore, there is a potential for antigen-loaded vesicles as an effective formulation for TCI.

In this study, for the first time these two approaches were combined, *i.e.* microneedle pretreatment and antigen-loaded vesicle formulations, to assess their potential in TCI, using DT (MW: 58 kDa; isoelectric point: 4.7) as an antigen. Two types of vesicles have been included. Cationic liposomes were made of soybean phosphatidylcholine (SPC) and 1, 2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP). Span 80 was incorporated to increase elasticity of the vesicle bilayer [24]. These liposomes in the absence or presence of Span 80 are referred to as DT-Lip and DT-ELip (DT Elastic Liposome), respectively. Characterization of their physicochemical properties is reported in this study. Surfactant-based vesicles were composed of sucrose-laurate ester (L-595) and sodium bistridecyl sulfosuccinate (TR-70). Octaoxyethylene-laurate ester (PEG-8-L) was used to increase the vesicle elasticity. This DT-containing vesicle

formulation has been developed and characterized in Chapter 5, and referred to as DT-Ves in this chapter. Selected formulations were applied in TCI onto intact or microneedle-treated skin of mice and compared with intradermal (i.d.) and s.c. immunization. Their immunogenicity *in vivo* was evaluated by measuring serum IgG (subtype) and neutralizing antibody titers. Their immune stimulatory properties were assessed *in vitro* using human peripheral blood mononuclear cell-derived immature DCs.

Materials and methods

Materials

SPC and DOTAP were kindly supplied by Lipoid GmbH (Ludwigshafen, Germany). Diphtheria toxin (batch 79/1), DT (batch 98/40, protein content 12.6 mg/ml by BCA assay, 1 µg equals to approximately 0.3 Lf), horse anti-DT and horseradish peroxidase (HRP) conjugated anti-DT were provided by The Netherlands Vaccine Institute (NVI, Bilthoven, The Netherlands). HRP-conjugated goat anti-mouse (HRP-GAM) IgG (γ-chain specific), IgG1 (γ1-chain specific) and IgG2a (γ2a-chain specific) were purchased from Southern Biotech (Birmingham, US). Adju-Phos® (alum) was obtained from Brenntag Biosector (Copenhagen, Denmark). Chromogen 3, 3', 5, 5'-tetramethyl-benzidine (TMB) and the substrate buffer were purchased from Biosource B.V. (Nivelles, Belgium). Tween 20, lyophilized bovine serum albumin, Folin Ciocalteu's phenol reagent, cholera toxin and Span 80 were ordered from Sigma-Aldrich (Zwijndrecht, The Netherlands). Tween 80 was purchased from Merck (Darmstadt, Germany). Ficoll and Percoll were ordered from GE Healthcare (Eindhoven, The Netherlands). Nimatek® (100 mg/ml ketamine), Rompun® (20 mg/ml xylasine) and the injection fluid (0.9% NaCl) were obtained from a local pharmacy. All other chemicals used were of analytical grade and all solutions were prepared with distilled water.

Methods

DT vesicle formulation preparation

The compositions of the DT vesicle formulations are listed in Table I. The DT-Lip and DT-ELip were prepared using the film rehydration and extrusion method. SPC, Span 80 and DOTAP, dissolved in chloroform, were mixed in an appropriate ratio and formed a thin film at the bottom of the flask using a

rotary evaporator. Residual organic solvent in the film was removed by 30 min nitrogen flow. The film was rehydrated by 10 mM phosphate buffer (PB, pH 7.4, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4) or 10 mM citrate buffer (CB, pH 5.0, 4.0 mM $H_3C_6H_5O_7$ and 6.0 mM $Na_3C_6H_5O_7$) with or without saline (153 mM NaCl, PBS or CBS) containing 1.5 mg/ml DT. The concentration of lipids in the buffer was 5% w/w. The resulting dispersions were shaken at 200 rpm in the presence of glass beads for 2 h at room temperature, followed by 2×15 s tip sonication (20% energy output, Branson Ultrasonics, Danbury, UK). The formulations were extruded (LIPEX™ Extruder, Northern Lipids Inc., Canada) 3 times through a pair of polycarbonate filters with pore size of 200 nm and 3 times through another pair with pore size of 100 nm (Nucleopore Millipore, Amsterdam, The Netherlands). DT-Ves were prepared and characterized in Chapter 5. Briefly, the surfactants were dissolved in ethanol, while TR-70 was dissolved in an ethanol/isopropanol mixture. After mixing in an appropriate ratio, the organic solvents were evaporated overnight in a vacuum centrifuge and rehydrated with 10 mM CB (pH 5.0) containing 1.5 mg/ml DT. Vesicles were formed after 3×5 s tip sonication (20% energy output, Branson Ultrasonics). Subsequently, the pH of the buffer was lowered to 4.5 by addition of 0.1 M HCl in order to associate DT with the vesicles. The final concentration of the surfactants in buffer was 10% w/w.

Table I. DT-loaded vesicle formulations

Buffer (pH)	Components and Molar Ratio:	Abbreviated name
	L-595:PEG-8-L:TR-70	
CB (4.5)	5:5:1	DT-Ves
	SPC:Span 80:DOTAP	
PBS (7.4)	9:0:1	DT-Lip-PBS
PBS (7.4)	9:3:1	DT-ELip-PBS
PB (7.4)	9:0:1	DT-Lip
PB (7.4)	9:3:1	DT-ELip
CBS (5.0)	9:0:1	DT-Lip-CBS
CBS (5.0)	9:3:1	DT-ELip-CBS
CB (5.0)	9:0:1	DT-Lip-5
CB (5.0)	9:3:1	DT-ELip-5

Size and ζ–potential measurements

The size and polydispersity index (PDI) of all formulations were measured by dynamic light scattering using a Zetasizer (Nano ZS, Malvern Ltd., United Kingdom). All size measurements were performed at 25°C at an angle of 173°

between the laser and the detector. Before measuring, all formulations were diluted in their corresponding buffer. Formulations were measured at regular time points during a period of two weeks after preparation to evaluate the colloidal stability. The ζ-potential was measured by laser Doppler electrophoresis using the same device. The formulations were stored at 4 ºC in between these measurements.

DT-vesicle association

To determine the DT-vesicle association ratios in the formulations, size exclusion chromatography was performed using a Sepharose® CL-4B (Amersham, Uppsala, Sweden) column as described in Chapter 5. Plain vesicles (cationic liposomes or anionic surfactant vesicles) and free DT in buffer solution served as controls to determine the time window for fraction collection. Each formulation was run with its corresponding buffer as mobile phase at a flow speed of 0.40 ml/min. The recovery rate of DT after SEC was determined by a modified Lowry-Peterson protein assay, which separates the antigen from the lipids/surfactants through a precipitation step and measures total protein content in each fraction [25]. The antigenicity of DT in each fraction was measured by ELISA, using horse anti-DT serum and a HRP-conjugated horse anti-DT, which measures the antigenicity of accessible DT in the presence of the vesicle components. Data were analyzed using the principle of parallel-line analysis of the OD-log concentration plots as described by Tierney *et al*. [26].

To examine the status of DT-liposome association when encountering physiological condition, the formulations were eluted in SEC using PBS. The resulting liposome associated-DT fractions were further treated by 1% Triton X-100 to disrupt the liposome structure and release the encapsulated DT. The antigenicity of DT in these fractions were determined by ELISA and compared to the corresponding fractions eluted by the original buffers.

Microneedle array and applicator

The microneedle array used in this study was manufactured from commercially available 30G hypodermic needles (Becton Dickinson, Alphen a/d Rijn, The Netherlands) as described previously [27]. The needles were assembled as a 300 μm-long, 4×4 array on a polymer back plate with a

surface area of about 0.5 cm^2. The microneedles were inserted into the skin at a speed of 3 m/s using the electric impact applicator.

Evaluation of vesicle elasticity

The elasticity of the DT-loaded vesicles was evaluated by extrusion through a modified Avanti Mini-Extruder® (Avanti Polar Lipids Inc., Delfzyl, The Netherlands). This method evaluates vesicle elasticity by comparing the extruded volume of dispersions under constant pressure as a function of time. In detail, the Mini-Extruder® was mounted on a scaffold with both syringes standing perpendicularly, donor above receptor. A polycarbonate filter with pore size of 30 nm was inserted in between the donor and acceptor syringes and 1 ml of vesicle dispersion was loaded in the donor syringe. A 1-kg weight was mounted to generate a constant pressure of about 10 bar on the piston of the donor syringe. Upon release of the weight, the extruded volume of the dispersion in the receptor syringe was recorded every minute over a 10-minute period. This procedure was repeated two more times using freshly prepared dispersions. The elasticity of vesicle membrane was calculated using the following formula as reported by Van den Bergh *et al.* [28].

$$D = J\left(\frac{r_v}{r_p}\right)^2$$

Where, D, elasticity index of vesicle membrane; J, volume of dispersion extruded over 5 min; r_v, size of vesicles; and r_p, pore size of the filter.

Immunization protocol

Female BALB/c mice (H2d), 8-week old at the start of the experiments, were purchased from Charles River (Maastricht, The Netherlands), and maintained under standardized conditions in the animal facility of the Leiden/Amsterdam Center for Drug Research, Leiden University. The study was conducted in conformity with the Public Health Service Policy on use of laboratory animals and had been approved by the Research Ethical Committee of Leiden University.

Three immunization studies were carried out. Negatively charged DT-Ves and positively charged DT liposome formulations were applied on intact or microneedle-treated skin in the first and second study, respectively.

Formulations were applied occlusively or non-occlusively, with the presence or absence of CT as an adjuvant. Immunization of DT-Lip *via* i.d. injection was carried out in the third study.

One day before immunization, the abdominal skin of mice of TCI groups was shaved and rested for 24 h. During immunization, mice were anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. The shaved skin area was wiped with 70% ethanol. For microneedle treatment, a skin fold was supported by styrofoam and pierced using the microneedle array right before the application of DT formulations. 70 µl DT formulations containing 100 µg DT (and 100 µg CT if applicable) per mouse were applied onto intact or treated skin. The formulations were carefully spread over the entire skin area of application, about 2 cm^2 restricted by a metal ring. After 1 h occlusive or non-occlusive incubation, the skin area was extensively washed with lukewarm tap water and patted dry twice. In the third study, 30 µl DT-Lip or DT solution containing 5 µg DT were given intradermally. 5 µg of DT adsorbed onto alum (DT-alum) in a 100 µl saline suspension was administered by s.c. injection for all three studies as a control. The DT-alum formulation was prepared as previously described and the adsorption of DT to alum was between 70% and 80% [29]. All DT formulations were freshly prepared before each vaccination. Mice were immunized at day 1, 21 and 42 (at approximately the same skin region for the TCI groups) and sacrificed at day 56. Blood was sampled from the tail vein one day before each immunization and whole blood was collected from the femoral artery during sacrifice. Cell free sera were obtained using MiniCollect® tubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) by centrifugation after clot formation and stored at -80 ºC until use.

Serum antibody assays

Serum IgG, IgG1 and IgG2a titers were determined by ELISA. Briefly, ELISA plates (Microlon®, Greiner Bio-one) were coated with DT at 4°C overnight. Two-fold serial dilutions of serum samples were applied in the plates and the containing DT-specific antibodies were detected by HRP-GAM IgG, IgG1 or IgG2a using TMB as substrate. Antibody titers are expressed as the reciprocal of the calculated sample dilution corresponding to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance-log dilution curve. Mice with serum sample unable to reach the half-saturated

absorbance value at the lowest (ten-fold) dilution were considered as non-responders.

Protective immunity against diphtheria depends on the presence of circulating neutralizing antibodies, evaluated using Vero cell test: the WHO standard method to assess the success of diphtheria vaccination, which relies on the inhibition of a cytotoxic dose of diphtheria toxin [30]. In brief, after complement inactivation, two-fold serial dilutions of serum samples were prepared with complete medium 199 (Gibco, Breda, The Netherlands) and applied to microtiter plates (CELLSTAR®, Greiner Bio-one). Subsequently, 2.5×10^{-5} Lf diphtheria toxin was added to each well. After 2 h incubation at 37 ºC for neutralization, Vero cell suspension was added to each well. Covered with a plate sealer, Vero cells were incubated at 37 ºC in 5% CO_2 for 6 days. The end point was taken as the highest dilution protecting the Vero cells.

DC maturation study

Immature DCs were prepared from human peripheral blood mononuclear cells, which were isolated by Ficoll-Percoll density-gradient centrifugation on day 0. Monocytes were separated from platelets due to their adherence to the plastic surface (24 well plate for cell culture, Costar®, Corning, New York, US) after incubation at 37 °C for 60 min. The adherent cells were cultured for 6 days in RPMI 1640, supplemented with 10% v/v fetal calf serum (FCS, Biosource-Invitrogen, Breda, The Netherlands), 1% glutamine, 100 U/ml penicillin and 0.1 mg/ml of streptomycin, 250 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Biosource-Invitrogen) and 100 U/ml interleukin-4 (IL-4, Biosource-Invitrogen) at 37°C with 5% CO_2 to differentiate into immature DCs. Medium was refreshed at day 3. At day 6, the medium was replaced by new medium containing GM-CSF and 2 µg/ml DT, either free, mixed with CT or associated in liposomes or vesicles, using lipopolysaccharide (LPS, from *Escherichia coli*, Sigma) as a positive control. Then DCs were incubated for 48 h at 37°C. Subsequently, DCs were washed 3 times with PBS containing 1% w/v BSA and 2% v/v FCS and incubated for 30 min with a mixture of 20x diluted anti-HLADR-FITC, anti-CD83-PE and anti-CD86-APC (Becton Dickinson) on ice. Cells were washed again and the expression of MHC II, CD83 and CD86 was quantified using flow cytometry (FACS Canto II, Becton Dickinson). The up-regulation of these three surface markers by 50 ng/ml LPS were set as 100%. Live cells were gated based on forward and side scatter. A minimum of 10,000 DC events were analyzed in

each experiment. The study was repeated using DCs from at least three different donors.

Statistical analysis

IgG (subtype) antibody titers were analyzed with two-way ANOVA with Bonferroni posttest, and the neutralizing antibody titers were analyzed using one-way ANOVA with the same posttest. Other analyses were performed where suitable as indicated. Statistical analysis was carried out using Prism Graphpad and a *p* value less than 0.05 was considered significant.

Results

Colloidal properties of DT vesicle formulations

Particle size and ζ-potential of the DT vesicle formulations are provided in Table II. Particle size and ζ-potential measured at day 7 and day 14 were very similar to the original values (data not shown), indicating good colloidal stability for all formulations listed. Particle sizes of the liposome formulations without Span 80 were smaller than those in the presence of Span 80. Formulations prepared by extrusion method show lower PDI than those prepared by sonication method.

Association of DT with the vesicles

The DT-Ves was characterized in Chapter 5 (Table II). The majority of DT was associated with vesicles when the pH of the buffer was lowered to 4.5 and dissociated when pH was increased to 7.4. This association and dissociation were not influenced by the ion strength of the buffer.

For the DT-containing cationic liposomes, when prepared in PB at pH 7.4 and CB at pH 5.0, high DT-liposome association was observed. After SEC of DT-Lip and DT-ELip, nearly all protein content was recovered from the liposome-associated DT fractions, whereas for DT-Lip-5 and DT-ELip-5 the recovery from the liposome-associated DT fraction was about 30% (Fig. 1a). However, when prepared in PBS and CBS instead, the liposome-associated DT in all four formulations appeared to be lower than 15% (Data not shown). This indicates that ionic strength and pH of the buffer play a prominent role in the antigen-liposome association. Formulations prepared in PB and CB were selected for further studies due to higher DT-liposome association.

Table II, Characterization of DT vesicle formulations. Data shown are mean ± SD of three different batches.

Abbreviated name	Size (nm)	PDI	ζ-potential (mV)	Association % [1] (Lowry-Peterson)	Encapsulation % [2] (ELISA) in PBS
DT-Ves	101 ± 6	0.28 ± 0.03	-77.3 ± 3.0	76.8 ± 2.9	-
DT-Lip	164 ± 2	0.23 ± 0.02	24.5 ± 1.4	86.7 ± 3.9	36.0 ± 4.5 [3]
DT-ELip	175 ± 3	0.21 ± 0.03	24.4 ± 0.6	86.7 ± 5.1	46.9 ± 4.3 [4]
DT-Lip-5	124 ± 9	0.12 ± 0.02	24.6 ± 2.0	29.9 ± 4.0	14.4 ± 4.0
DT-ELip-5	149 ± 3	0.11 ± 0.01	24.8 ± 1.1	40.0 ± 4.3	13.0 ± 3.3

[1]Associated DT: includes the surface-attached DT, bilayer-intercalated DT and the liposome-encapsulated DT
[2]Encapsulated DT: DT released upon Triton x-100 treatment, detected by ELISA
[3]Significantly high than those of DT-Lip-5 (p<0.001, t-test)
[4]Significantly high than those of DT-ELip-5 (p<0.001)

When detected with ELISA, the percentages of DT antigenicity in the free-DT fractions were similar to those of protein content, while the antigenicity from the liposome-associated DT fractions was much less (Fig. 1b). This significant loss of DT antigenicity is probably due to the shielding effect of the liposome on the encapsulated DT. Selected formulations were eluted with PBS (pH 7.4) in SEC to examine the status of DT-liposome association in physiological condition. DT antigenicity of the resulting fractions were measured and compared with those eluted by CB/PB (Fig. 1c and 1d). These figures show that part of the liposome-associated DT, probably attached to the liposome surface, was dissociated from the liposomes. When the liposome-associated DT fractions were further treated with Triton X-100, increased DT antigenicity was detected and the overall recovery rate of DT antigenicity was increased to about 80%, comparable to the control group. This increased DT antigenicity was previously shielded by liposome and represents the encapsulated DT under physiological condition (Table II). The DT-encapsulation ratio in PB at pH 7.4 was significantly higher than that in CB at pH 5.0 (p<0.001, t-test), most likely due to the higher negative charge of DT at pH 7.4, which facilitates the electrostatic interaction between DT and the liposome components.

Elasticity of the DT-loaded vesicles

The extruded volumes of DT-loaded vesicle formulations and the calculated elasticity indices are shown in Figure 2a and 2b, respectively. The unloaded surfactant vesicles showed relatively high elasticity. However, it was dramatically reduced by the loading of DT. When 0.2 mg/ml or more DT was incorporated, the dispersions could hardly be extruded, similar to the rigid

DT-Lip. On the other hand, DT-ELip retained its elasticity and can be extruded through filter with 30 nm pore size. The particle size of DT-ELip (~175 nm) was much larger than that of empty vesicles (~100 nm). For this reason, even though its extrusion speed was slightly slower than the empty surfactant vesicles, it shows the highest elasticity index.

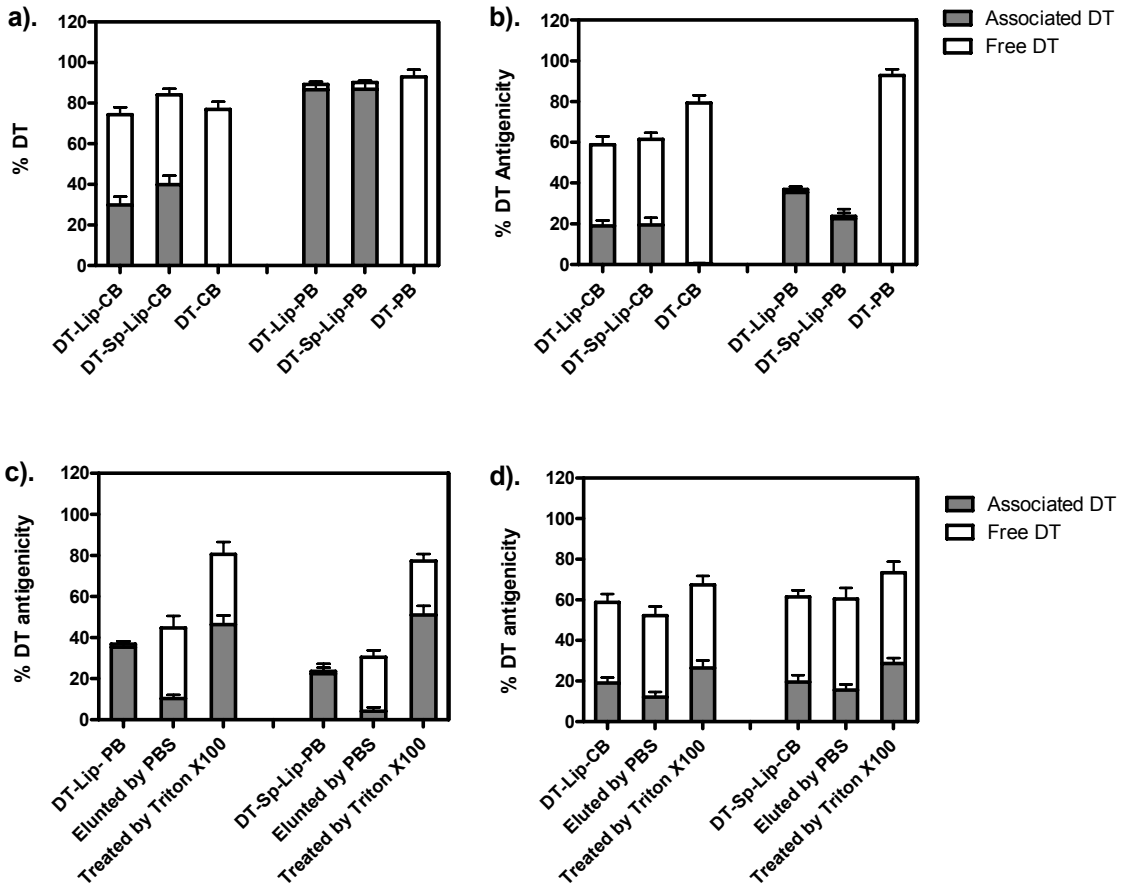

Figure 1. DT-liposome association in formulations prepared in CB or PB. The liposome-associated DT and free DT were separated through SEC fractionation of formulations by their corresponding buffers and determined with Lowry-Peterson protein assay (1a) and ELISA (1b), free DT in PB and CB served as control. DT liposome formulations prepared in CB and PB were eluted in SEC with PBS (referred to as “eluted by PBS”, 1c and 1d). The resulted liposome-associated DT fractions were further treated with 1% Triton X-100 to release the encapsulated DT and both were measured with ELISA (referred to as “treated by Triton X-100”). Data are shown as mean + SD of three different batches.

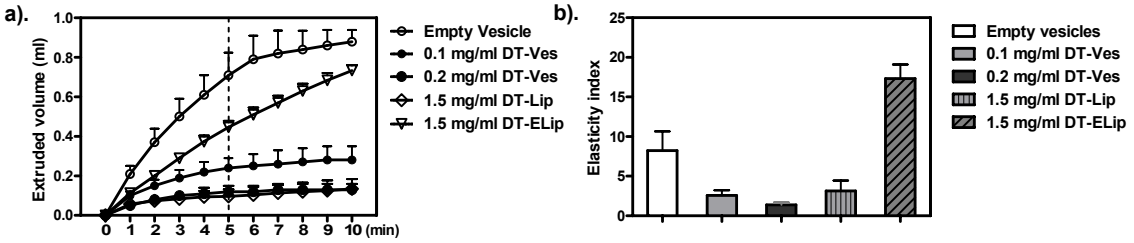

Figure 2. The vesicle elasticity measured with the extrusion method. DT vesicle formulations were extruded through polycarbonate filters with pore size of 30 nm under a constant pressure of 10 bar. The extruded volumes of these dispersions were recorded once per minute during 10 min (2a). Particle sizes of the dispersions were measured by DLS and the elasticity indices were calculated using the extruded volumes after 5 min (2b). Data shown are mean + SD of three batches.

Analysis of antibody titers induced in TCI

DT-loaded anionic surfactant vesicles

Firstly, TCI was performed by applying free DT and DT-Ves onto microneedle-treated or intact skin using CT as an adjuvant. The serum IgG titers after prime, the first boost and the second boost and the neutralizing antibody titers after the second boost through occlusive application are provided in Figure 3. Non-occlusive application of the same formulations was also studied, and no significant difference was found compared to the corresponding occlusive groups. For clarity, only data from non-occlusive application of DT-Ves onto intact skin are shown in Figure 3b. Pretreatment of the skin using the microneedle array improved the antibody responses drastically for both free DT and DT-Ves (Fig. 3a *vs.* 3b). The presence of CT further potentiated the IgG titers for DT-Ves ($p>0.05$, $p<0.001$ and $p<0.001$ after prime, the first boost and second boost, respectively) and for free DT ($p<0.05$, $p<0.001$ and $p<0.05$ after prime, the first boost and second boost, respectively). For DT-Ves, IgG titers were very close to those induced by s.c. DT-alum after the second boost ($p>0.05$); whereas for free DT, comparable IgG titers were obtained from the first boost on ($p>0.05$). Surprisingly, DT-Ves formulations did not induce higher IgG titers than free DT through either occlusive or non-occlusive application ($p>0.05$ for all three time points). The IgG1 followed the IgG and showed slightly lower titers with similar trend, while the IgG2a titers were much less developed with many non-responders (data not shown).

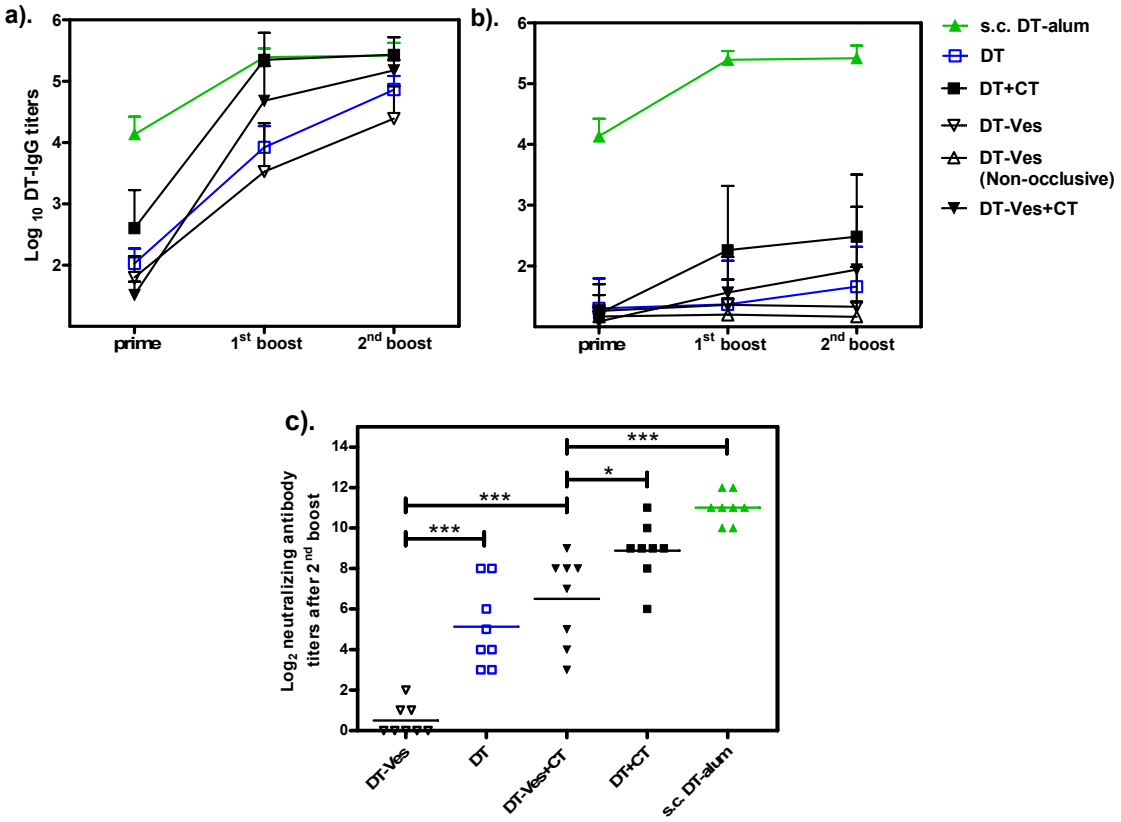

Figure 3. DT-specific IgG titers and neutralizing antibody titers after TCI of DT-Ves and free DT using CT as an adjuvant. Formulations were applied occlusively (unless specified in figure 3b) on microneedle-treated (3a) or intact skin (3b) at day 0, 21 and 42, s.c. injection of DT-alum served as control. Sera were collected after prime, the 1st boost and the 2nd boost (day 20, 41 and 55) and IgG titers were determined with ELISA. Data shown as mean + SD (n=8). Non-responders were given an arbitrary log-value of 1. Neutralizing antibody titers of TCI on microneedle-pretreated skin after the 2nd boost were evaluated with Vero cell test. An arbitrary log-value of 0 was given when titers were below detection limit (3c). Neutralizing antibody titers from intact skin were all below detection limit and therefore not shown (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

The neutralizing antibody titers showed the same trend as the IgG titers after the second boost (Fig. 3c). The presence of CT enhanced the titers of both free DT and DT-Ves ($p<0.001$ for both). The differences between free DT and DT-Ves are more pronounced. DT-Ves induced lower titers than DT ($p<0.001$ and $p<0.05$ for with and without CT, respectively). The titers induced by DT-Ves with CT were lower than those of s.c. DT-alum ($p<0.001$), whereas for IgG titers, the difference was not significant. In the presence of CT, free DT induced comparable neutralizing antibody titers to those of s.c. DT-alum, the same as the IgG titers.

In TCI on intact skin, free DT and DT-Ves resulted in neither substantial antibody responses nor detectable neutralizing antibody titers. DT-Ves did not improve the immune responses of free DT, either.

As DT is released quickly from DT-Ves at pH 7.4, and the negative surface potential may reduce the interactions between vesicles and the cell membranes of APCs, positively charged vesicles were included in our studies. Initially we focused on developing positively charged, DT-loaded, surfactant vesicles to obtain high DT-vesicle association at pH 7.4. However, by replacing TR-70 with DOTAP or dimethyl dioctadecyl ammonium as the charge inducer, stable formulations could not be obtained. The cationic liposome formulations were developed instead; in which considerable amount of DT has stable association with liposomes when diluted in PBS.

DT-loaded cationic liposomes

On microneedle-treated skin, TCI was performed using DT-Lip, DT-Lip-5 and DT-ELip using CT as an adjuvant. Only occlusive application was performed as no differences between occlusion and non-occlusion were observed in the study of DT-Ves. Serum IgG and neutralizing antibody titers are provided in Figure 4. The presence of CT potentiated the IgG titers and neutralizing antibody titers of DT-Lip significantly (p<0.001 for IgG titers after the first and second boost, and the neutralizing antibody titers, Fig. 4b and 4c). However, the DT liposome formulations did not induce higher immune responses than free DT, either. DT-ELip resulted in lower IgG titers after prime and the first boost, and lower neutralizing antibody titers after the second boost as compared to free DT (p<0.01, p<0.001 and p<0.01, respectively). Only after the second boost, it induced a comparable level of IgG titers as free DT (p>0.05), higher than those of DT-Lip-5 and DT-Lip (p<0.05 and p<0.001, respectively, Fig. 4a). DT-Lip and DT-Lip-5 induced lower IgG titers than free DT at all three time points (p<0.05, p<0.001 and p<0.001, respectively, Fig. 4a) and lower neutralizing antibody titers (p<0.001, Fig. 4c). Similar titers induced by DT-Lip and DT-Lip-5 indicate that the immunogenicity is not sensitive to the different DT-liposome association ratios (Fig. 4a). In accordance to the study of DT-Ves, the IgG1 titers of DT liposome formulations followed the IgG titers and showed a similar trend. The IgG2a titers were much less developed (data not shown).

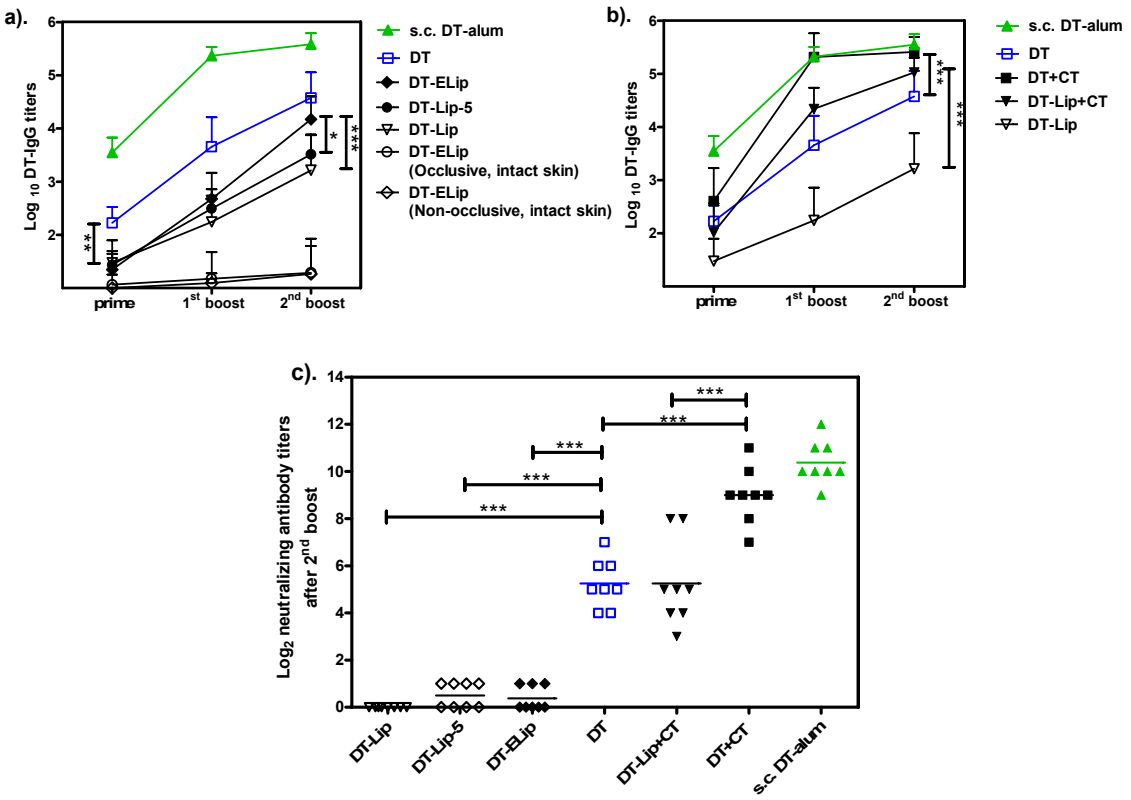

Figure 4. DT-specific IgG titers and neutralizing antibody titers induced by DT liposome formulations in TCI. Formulations were applied occlusively on microneedle-treated skin (unless specified in figure 4a) at day 0, 21 and 42, s.c. injection of DT-alum as control. Sera were collected after prime, the 1st boost and the 2nd boost (day 20, 41 and 55) and IgG titers were determined with ELISA (4a and 4b). Data shown as mean + SD (n=8). Non-responders were given an arbitrary log-value of 1. Neutralizing antibody titers after the 2nd boost were evaluated with Vero cell test (4c). An arbitrary log-value of 0 was given when titers were below detection limit (*: p<0.05, **: p<0.01, ***: p<0.001).

As for DT-Ves, the loss of vesicle elasticity by the loading of DT was also suspected to account for its low immunogenicity on intact skin, TCI of DT-ELip on intact skin was included because of its superior elasticity. However, the IgG levels induced were low and comparable to those of free DT and DT-Ves (Fig. 4a *vs.* Fig. 3); and occlusive and non-occlusive applications showed no difference in serum IgG titers (Fig. 4a).

Analysis of antibody titers induced via i.d. immunization

All the data suggest that vesicle formulations do not improve the immune response in TCI. To study whether differences in delivery efficiency across the skin barrier play a role, DT-Lip and free DT were administered *via* i.d. injection. The resulting serum IgG, IgG1, IgG2a and neutralizing antibody

titers are shown in Figure 5. The IgG, IgG1 and neutralizing antibody titers from free DT and DT-Lip were not significantly different. Unlike TCI, i.d. immunization of free DT and DT-Lip induced substantial IgG2a titers. Interestingly, DT-Lip induced a faster increase in IgG2a titers than free DT and significantly higher IgG2a titers were found after the first and the second boost (Fig. 5b, $p<0.001$, two-way ANOVA), suggesting immune modulation to a more Th1 biased response.

DC maturation upon contact with DT-loaded vesicles

To circumvent the complicated factors in delivery efficiency across the skin barrier, the direct stimulatory effects of DT formulations on the maturation of DCs were investigated *in vitro*. The up-regulation of the surface markers, MHC II, CD83 and CD86 was determined after culturing immature DCs in the presence of the DT formulations (Fig. 6). DT alone had no effect on the surface markers tested. CT showed the most potent stimulation to the immature DCs. It significantly up-regulated MHC II expression to levels higher than LPS and at the same time moderately enhanced those of CD86 as compared to free DT ($p<0.001$ and $p<0.01$, respectively, one way ANOVA). DT-Ves, DT-Lip and DT-ELip didn't show significant up-regulation to all three surface markers as compared to free DT.

Discussion

In the current study, stable DT-loaded liposome formulations were prepared and characterized in terms of their particle size, ζ-potential, degree of DT-vesicle association and vesicle elasticity. Two types of DT vesicle formulations, cationic liposomes and anionic surfactant vesicles, were studied in TCI and the skin was either pretreated with microneedle arrays, or left intact. Pretreatment with the microneedle array and co-administration with CT improved the immune responses of all of the formulations dramatically. However, the immunogenicity was not improved further by using vesicle formulations.

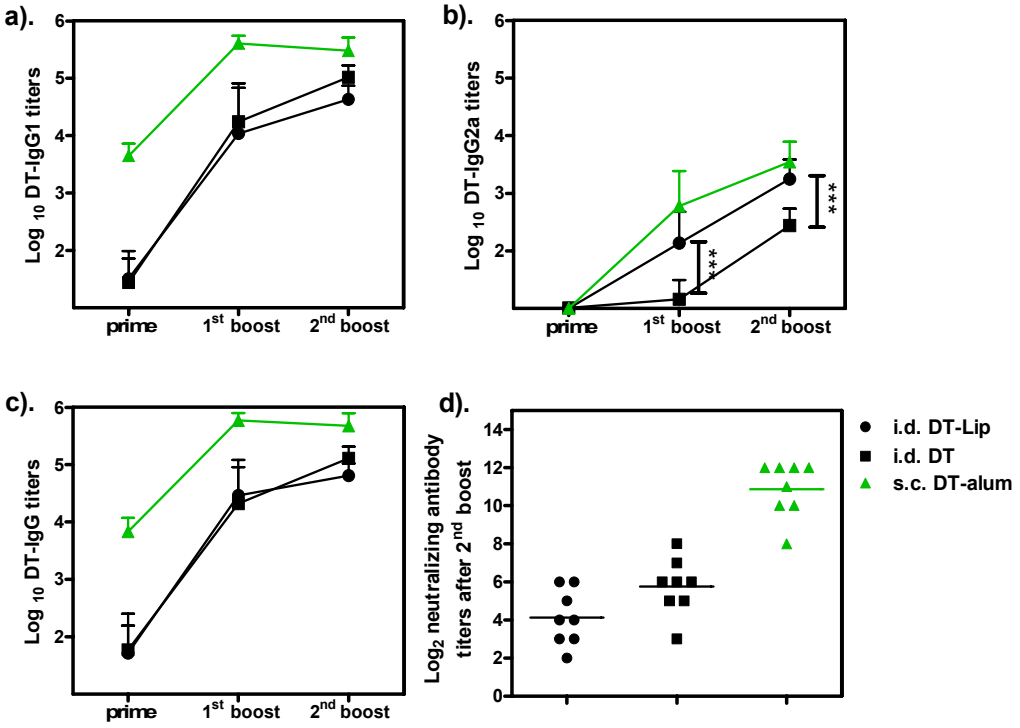

Figure 5. DT-specific IgG subtype antibody titers and neutralizing antibody titers after i.d. injection of DT-Lip-PB and free DT. Vaccination was performed at day 0, 21 and 42, s.c. injection of DT-alum was as control. Sera were collected after prime, the 1st boost and the 2nd boost (day 20, 41 and 55) and IgG, IgG1 and IgG2a titers were determined with ELISA (5a, 5b and 5c). Data shown as mean + SD (n=8). Non-responders were given an arbitrary log-value of 1. Neutralizing antibody titers after the 2nd boost were evaluated with Vero cell test (5d). An arbitrary log-value of 0 was given when titers were below detection limit (***: p<0.001).

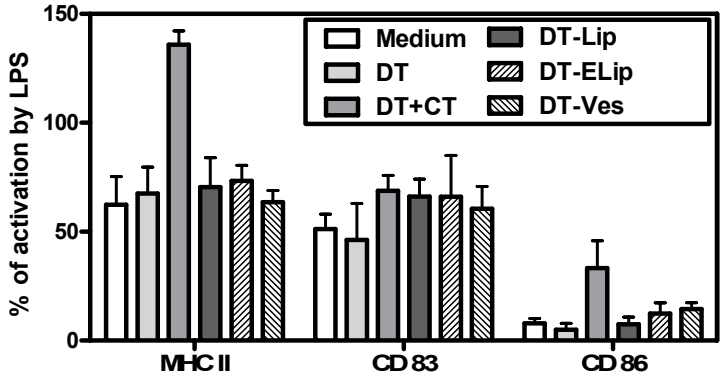

Figure 6. Stimulatory effects of DT formulations on immature DCs as indicated by the up-regulation of the surface markers; MHC II, CD83 and CD86. The up-regulations of these three surface markers by 50 ng/ml LPS were set as 100%. The culture medium has a basal level of stimulation to the DCs as it contains GM-CSF. The final concentrations of each formulation component in culture medium were: DT (2 μg/ml), DT+CT (2 μg/ml DT with 2 μg/ml CT), DT-Lip-PB and DT-Sp-Lip-PB (2 μg/ml DT with 70 μg/ml total lipids), DT-Ves (2 μg/ml DT with 140 μg/ml total surfactants). Data shown are mean + SD (n ≥ 3).

Initially, surfactant vesicles were chosen to formulate DT for TCI as a vaccine delivery system, as they have been shown to enhance the transport of small molecule drugs across human skin. Evidence for an exceptional interaction between these vesicles and human skin *in vivo* was obtained by freeze fracture electron microscopy and Fourier transform infrared studies [23, 31]; vesicular structures were observed in the deep layers of the *stratum corneum* after one hour non-occlusive application. However, there was no evidence of vesicle material in the viable epidermis. The same vesicle system was employed for DT delivery in the current study. However, on intact skin, no substantial immune response was induced by DT-Ves and free DT, following either occlusive or non-occlusive application. The comparable immunogenicity of DT-Ves and free DT on intact skin indicates that vesicles do not efficiently transport the antigen across the *stratum corneum*. As this could be due to fast DT release from vesicles at pH 7.4 and the loss of vesicle elasticity when DT is incorporated, it was decided to prepare DT-ELip, which showed association of DT with the vesicles/liposomes at pH 7.4 and increased elasticity. However, in the subsequent *in vivo* immunization study, DT-ELip did not enhance the immunogenicity of topically applied DT on intact and microneedle pretreated skin. As the composition of the Span liposomes is very similar to that of Transfersomes® used in TCI for hepatitis B surface antigen and gap junction protein, the outcome of our studies are in contrast to those reporting that ultradeformable vesicles induce potent immune responses on intact skin after non-occlusive application [19, 32]. In our hands, elastic vesicles enhance the transport of small molecules across the skin [21-23], but do not improve the immunogenicity of topically applied antigens, *e.g.* DT. In addition, there is no difference between occlusive and non-occlusive application for the elastic liposomes. Therefore, the osmotic gradient does not seem to play a central role as a driving force for antigen diffusion using these formulations.

To facilitate the transport of antigens into the viable epidermis, inclusion of skin barrier disruption methods, *e.g.* microneedle array pretreatment, tape striping or electroporation *etc.*, appears to be crucial [33]. Microneedle pretreatment has been shown to enhance the transcutaneous transport significantly. Henry *et al.* demonstrated four orders of magnitude increase in permeability for calcein and BSA through human epidermis *in vitro* after pretreatment with a microneedle array of 150 µm needle length [9]; while Verbaan *et al.* showed penetration of 72 kDa dextran across microneedle-pretreated human skin *in vitro* using 300 µm long microneedle

array [27]. In the current study, the improved immunogenicity provided by the microneedle pretreatment may be attributed not only to enhanced antigen transport across the skin barrier, but also to the danger signals (IL-1α, IL-1β, GM-CSF and TNF-α released from the surrounding keratinocytes) caused by the skin barrier disruption, which sensitize and mobilize the skin APCs [34].

The induced immune response depends on both the permeation along the conduits and the interaction of the formulation with the skin APCs. I.d. vaccination was performed to exclude the influence of transport along the conduits on the immune responses. In addition, the DC study provided equal contact of DT to the APCs *in vitro*. I.d. vaccination of free DT and DT-Lip induced similar IgG1 and neutralizing antibody titers. This indicates that liposomes do not stimulate DCs. When comparing i.d. vaccination with TCI on microneedle-pretreated skin, where the same dose of DT in DT-Lip induced significantly lower immune response than free DT, it is clear that the transport of DT-loaded liposomes along the conduits is one of the limiting factors. This might be due to non-specific bindings of the positively charged liposomes to other cell membranes and negatively charged intercellular proteins, as reported by Yan *et al.* [35]. As the negatively charged vesicles also reduced the immune response, it seems that a lower diffusion rate along the conduits may also play a role. An application period longer than one hour may enhance the diffusion and improve the immune responses in TCI, as the conduits formed by the microneedles remain open for at least a few hours as reported by Bal *et al.* [36] and Banga *et al.* [37].

In TCI on microneedle-pretreated skin, it is notable that free DT induced faster IgG development than DT liposome formulations (Fig. 4). Most probably the soluble antigen diffuses faster along the conduits into the skin, and arrives earlier in the viable layers and the peripheral draining lymph nodes. The role of direct lymphatic drainage of free antigen and the subsequent capture by blood-derived, lymph node-resident DCs for presentation to T cells has been highlighted in recent studies [38-41]. So far, a DT/CT mixture, soluble antigen with soluble adjuvant, is the most immunogenic formulation in TCI on microneedle-pretreated skin. CpG ODN also demonstrated potent immune potentiation and modulation properties in our previous TCI study in Chapter 4. Moreover, the alum-adsorbed DT, big particles of several microns, reduced IgG titers dramatically and diminished the neutralizing antibody titers to undetectable level when applied on microneedle-treated skin (data not shown). Therefore, including other potent and soluble adjuvants is one of the

first directions for further optimization of TCI formulations. Antigen-adjuvant conjugates and antigen-adjuvant fusion constructs may also be potential candidates. Furthermore, it has been reported that following i.d. injection in mice, 20 nm particles are taken up into the lymphatics more readily than 45 or 100 nm particles and are retained in the lymph nodes for a longer time (up to 120 h) [42]. These ultra small particles as well as some specific types of vesicles, *e.g.* ISCOMS, virus-like particles and surface-modified liposomes [43, 44], hold promise in TCI when combined with microneedle array pretreatment. Last but not least, instead of relying on passive diffusion following microneedle treatment, the development of a microneedle array through which the vaccine formulation could be injected would provide more precise dose control and improve the vaccination efficiency.

Conclusion

We have demonstrated in the current study that the microneedle array pretreatment of the skin and co-administration of CT significantly enhances the immunogenicity of topically applied DT. However, formulating the antigen with vesicles, rigid or elastic, anionic or cationic, applied occlusively or non-occlusively, does not further improve the immune responses.

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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 sulfo succinate. 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.* ζ-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

[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.

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Appendices

List of abbreviations:

Ac:	Sodium acetate buffer
Alum:	Adju-Phos®, colloidal aluminum phosphate
APC:	antigen-presenting cell
BSA:	bovine serum albumin
CB:	citrate buffer
CBS:	citrate buffered saline
CLR:	c-type lectin-like receptor
CCL:	CC chemokine ligand
CM199:	complete medium 199
CRM 197:	cross-reacting material of diphtheria toxin
CSSS:	cyanoacrylate skin surface stripping
CT:	cholera toxin
CTA:	cholera toxin A subunit
CTB:	cholera toxin B subunit
CXCL:	C-X-C motif ligand
DAMP:	danger-associated molecular patterns
DC:	dendritic cell
DC-SIGN:	DC-specific ICAM-3-grabbing non-integrin
DDA:	dimethyl dioctadecyl ammonium
DLS:	dynamic light scattering
DOTAP:	1, 2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DT:	diphtheria toxoid
DTV:	diphtheria toxoid vesicle formulation
EPI:	epidermal powder immunization
ELISA:	enzyme-linked immunosorbent assay
FCA:	Freund's complete adjuvant
FCS:	fetal calf serum
GJP:	gap junction protein
GM-CSF:	granulocyte-macrophage colony-stimulating factor
HB-EGF:	heparin-binding epidermal growth factor precursor
HBsAg:	hepatitis B surface antigen
HI:	hemagglutination inhibition
HRP-GAM:	horseradish peroxidase conjugated goat anti-mouse
HSA:	human serum albumin
HSP:	heat shock protein
ICAM:	intercellular adhesion molecule-3

i.d.:	intradermal
IL-4:	interleukin-4
i.m.:	intramuscular
i.p.:	intraperitoneal
LC:	Langerhans cell
L-595:	sucrose-laurate ester
LPS:	lipopolysaccharide
MEA:	microenhancer array
MHC:	major histocompatibility complex
MN:	microneedle array
MPL:	monophosphoryl lipid A
Nalp3:	NACHT-LRR-PYD-containing protein
NLR:	NOD like receptor
NOD:	nucleotide oligomerization domain
NSAIDs:	non-steroidal anti-inflammatory drugs
OPD:	o-phenyldiaminedihydro-chloride
OPM:	o-palmitoyl mannan
PAMP:	pathogen-associated molecular patterns
PB:	phosphate buffer
PBMC:	peripheral blood mononuclear cell
PBS:	phosphate buffered saline
PBST:	phosphate buffered saline containing 0.05% Tween 20
PDI:	polydispersity index
PEG-8-L:	octaoxyethylene laurate ester
PRR:	pathogen recognization receptor
RIG:	retinoic acid inducible gene
RLR:	RIG-I-like receptor
s.c.:	subcutaneous
SEC:	size exclusion chromatography
Span 60:	sorbitan monostearate
Span 80:	sorbitan monooleate
Span 85:	sorbitan trioleate
SPC:	soybean phosphatidyl choline
TCI:	transcutaneous immunization
TEWL:	trans-epidermal water loss
TMB:	3, 3', 5, 5'-tetramethylbenzidine
TNF-α:	tumor necrosis factor-α
TR-70:	sodium bistridecyl sulfo succinate

TT:	tetanus toxoid
VLP:	virus-like particle
WIV:	whole inactivated virus

Transcutane subunit vaccin toediening

Een gecombineerde benadering van vesiculaire formuleringen en matrices van micronaalden

Samenvatting

Het onderzoek beschreven in dit proefschrift is gericht op dermale vaccinatie, d.w.z. vaccinatie via de huid.

De natuurlijke functie van de huid is de barrièrefunctie. Die wordt vooral gevormd door de buitenste dode huidlaag, de zogenaamde hoornlaag. In de daaronder gelegen levende huid bevinden zich o.a. dendritische cellen (DCs), die een cruciale rol spelen bij het op gang brengen van de immuunrespons. Dermale vaccinatie is vooral attractief omdat dit pijnloos kan gebeuren omdat pijnzenuwen afwezig zijn in het buitenste deel van de huid en omdat de concentratie DCs in de huid hoog is. Een hoge concentratie DCs maakt het mogelijk om efficient te vaccineren. Echter de grootste uitdaging is om het antigeen (het actieve gedeelte van het vaccin) in voldoende mate door de hoornlaag te transporteren in de richting van de DCs.

In het onderzoek beschreven in dit proefschrift werden zowel micronaalden als vesiculaire formuleringen gebruikt om het transport van het antigeen door de hoornlaag te verhogen. Micronaalden zijn naalden korter dan 1 mm met een diameter van 300 µm of kleiner. De voor dit proefschrift gebruikte naalden zijn massief en worden gebruikt om de huid vóór te behandelen waarna het vloeibare vaccin wordt opgebracht. Vesiculaire formuleringen bevatten deeltjes met een waterige kern omsloten door een hydrofobe laag van oppervlakte-actieve stoffen. De vesicles zijn, afhankelijk van hun samenstelling, elastisch en de penetratie van stoffen door de huid bevorderen. Tevens werden aan de formuleringen adjuvantia toegevoegd om de immunogeniciteit te verhogen.

Om het transport van het antigeen door de huid te bevorderen werd eerst het gebruik van micronaalden onderzocht, waarbij kleine gaatjes geprikt worden, zodat de barrierefunctie van de huid vermindert en het antigeen efficienter door de hoornlaag getransporteerd wordt in de richting van de DCs. Eerst werd onderzocht of de micronaalden gaatjes veroorzaken in de huid. Dit

onderzoek staat beschreven in ***hoofdstuk 3***. Micronaalden werden getest met een lengte varierend tussen 300 en 900 µm. De naaldjes werden gemonteerd in een plaatje, zodanig dat er 16 naaldjes in een 4x4 geometrie gerangschikt zijn. Het bleek dat micronaaldjes met een lengte van minstens 550 µm gaatjes in de huid veroorzaken, terwijl de kortere naaldjes niet noemenswaardig in de huid prikten. Niet alleen de naaldlengte maar ook de snelheid waarmee de naaldjes de huid raken bepaalt of de hoornlaag gepenetreerd wordt. Bij te lage snelheid rekt de elastische huid mee en vouwt zich om de micronaalden. Daarom werd een speciale electrische applicator ontwikkeld waarmee de naalden met constante, relatief hoge snelheid gebruikt kunnen worden. Het bleek inderdaad dat met behulp van de applicator een array met kortere micronaalden (245-300 µm lang) de muizenhuid kan penetreren op een uniforme en reproduceerbare manier. Dermale vaccinatie met difterie toxoid en een influenza antigeen werd uitgevoerd met cholera toxine (CT) als adjuvans. Hiertoe werd de huid al dan niet voorbehandeld met de micronaalden, waarna vervolgens de formulering opgebracht werd. In geval van difterie toxoid, was de voorbehandeling met de micronaalden noodzakelijk om substantiële immunogeniciteit te bereiken. Vergeleken met dermale toediening zonder voorbehandeling, zorgde de micronaalden voorbehandeling voor een 1000-voudige verhoging in immunogeniciteit. Toevoeging van CT verhoogde de immuunreactie verder tot een niveau dat vergelijkbaar was met de immuunrespons na subcutane toediening van difterie toxoid geadsorbeerd aan aluminiumfosfaat, de postieve controle. Met het influenza antigeen werden andere resultaten bereikt. De voorbehandeling met micronaalden zorgde niet voor een significant effect, maar de toevoeging van CT verhoogde de immunogeniciteit sterk. Deze resultaten tonen aan dat het effect van de voorbehandeling met micronaalden voor dermale vaccinatie afhankelijk is van het gebruikte antigeen. Er werd besloten in alle vervolgstudies difterie toxoid te gebruiken.

Vaccins, die toegediend worden via de huid, worden opgenomen door andere klassen DCs dan na conventionele intramusculaire of subcutane injecties. Daarom werd de invloed van verschillende adjuvantia op het type afweerreactie na dermale toediening met difterie toxoid onderzocht. Deze studies staan beschreven in ***Hoofdstuk 4***. De immunogeniciteit werd verhoogd door de voorbehandeling met micronaalden. De toevoeging van een adjuvans verhoogde de immunogeniciteit verder in deze volgorde: Quil A < CpG < CT. Het gebruik van adjuvantia had ook invloed op het type respons. De verhouding van de zogenaamde Th1/Th2 reactie nam toe in de volgorde:

geen adjuvans, Quil A, CT en CpG. Dit suggereert dat de immunogeniciteit, die opgewekt wordt door enkel difterie toxoid en die neigt naar een Th2 reactie, kan overgaan naar een Th1 reactie, afhankelijk van het gebruikte adjuvans. Hiermee kan de immunogeniciteit dus gemoduleerd worden.

In de literatuur staat beschreven dat elastische vesicles (deeltjes met een waterige kern omsloten door een hydrofobe laag van oppervlakte actieve stoffen) op een efficiënte manier medicijnen met een laag molecuulgewicht over de huid kunnen transporteren. In ***Hoofdstuk 5*** werd daarom een difterie toxoid bevattende vesiculaire formulering ontwikkeld en gekarakteriseerd. De vesicles bestaan uit uit sucrose-lauraat ester en natrium-bistridecyl sulfo-succinaat. Octa-oxyethyleen lauraat ester werd toegevoegd om de elasticiteit van de vesicles te verhogen. De variabelen voor de formuleringen waren de volgende: de verhouding van de verschillende bestanddelen, difterie toxoid concentratie, zuurtegraad (pH) en ionsterkte. De formuleringen werden geoptimaliseerd wat betreft de colloïdale stabiliteit en difterie toxoid-vesicle associatie. De pH had een dramatisch effect op de difterie toxoid-vesicle associatie. Bij pH 4.5 was meer dan 70% van het eiwit geassocieerd met de vesicles, terwijl minder dan 20% geassocieerd was bij pH 5.0. Hydrofobe interacties speelden een belangrijke rol bij deze associatie en de structurele integriteit van difterie toxoid bleef behouden tijdens de bereiding van de formulering. Omdat elastische vesicles voor laag moleculaire farmaca alleen efficient het transport door de huidbarriere verhogen indien de farmaca geassocieerd zijn met de vesicles, werd besloten om de formulering met een pH van 4.5 te selecteren voor in vivo studies.

Hoofdstuk 6 beschrijft een benadering, waarbij de voorbehandeling van micronaalden werd gecombineerd met vesiculaire formuleringen, die het antigeen bevatten. Dermale vaccinatie van muizen werd uitgevoerd d.m.v. het occlusief (geen verdamping van water mogelijk) of niet-occlusief (wel verdamping van water) aanbrengen van de formulering op de intacte of op de met micronaalden voorbehandelde huid. Uit de resultaten bleek dat difterie toxoid in vesicles niet tot een verbeterde immunogeniciteit leidde ten opzichte van vrij difterie toxoid. In de daaropvolgende studies bleek dat difterie toxoid de elasticiteit van de vesicles sterk verminderde. Dit verlies in elasticiteit en de snelle afgifte van difterie toxoid uit de vesicles bij pH 7.4 (de pH in de huid) zouden de oorzaak kunnen zijn voor de relatief lage immunogeniciteit. Daarom werden er positief geladen liposoom (vesicles bestaande uit voornamelijk fosfolipiden) formuleringen ontwikkeld, waarbij difterie toxoid

(negatief geladen) nog steeds met de liposomen geassocieerd is bij een pH van 7.4. De fysisch-chemische en de colloïdale eigenschappen, van de liposomen werden onderzocht en geoptimaliseerd, zoals dit ook was uitgevoerd voor de difterie toxoid-vesicles. Bij pH 7.4 bleek dat difterie toxoid voornamelijk geassocieerd was met de liposomen dankzij de elektrostatische interacties. Toevoeging van Span 80 verhoogde de elasticiteit van de liposomen in de aanwezigheid van difterie toxoid terwijl de associatie van difterie toxoid met de liposomen intact bleef. Echter uit de daaropvolgende dermale vaccinatie studie bleek dat difterie toxoid geformuleerd in positief geladen vesicles ook niet tot een verhoogde immunigeniteit leidde ten opzichte van vrij difterie toxoid. Dit gold voor zowel de intacte als met micronaalden voorbehandelde huid. Uit vervolgstudies bleek dat liposomen geen immuunstimulerende eigenschappen vertoonden wanneer ze werden toegevoegd aan DCs in vitro. Mogelijk is een verminderd transport over de huid de oorzaak van de lagere immunogeniciteit na dermale vaccinatie ten opzichte van vrij difterie toxoid, waarbij liposomen op zich niet de immunogeniciteit verhogen. Het inbouwen van adjuvantia in liposomen kan daar in de toekomst mogelijk verandering in brengen.

Uit het beschreven onderzoek blijkt dat de toepassing van vrij antigeen (difterie toxoid en het influenza antigeen) en difterie toxoid-bevattende vesiculaire formuleringen op intacte huid geen immuunrespons veroorzaakt. Dermale vaccinatie met het influenza antigeen is efficienter door toevoeging van CT, onafhankelijk van voorbehandeling met micronaalden. Wat betreft difterie toxoid, is voorbehandeling met micronaalden efficient. De immunogeniciteit kan verder verhoogd worden door gebruik van adjuvantia. difterie toxoid associatie met vesicles blijkt minder efficient te zijn om de immunogeniciteit te verhogen. Dit geld voor zowel onbehandelde huid als micronaalden behandelde huid. De sterkte en de kwaliteit van de afweerreactie na dermale toediening kan verder worden geoptimaliseerd door het gebruik van adjuvantia.

透皮免疫

纳米颗粒载体剂型和微针阵列的综合应用

本论文研究透皮免疫，即经由皮肤的疫苗接种。皮肤是人体的物理屏障。这一功能主要是由皮肤最表层的死细胞层，即角质层来执行的。角质层下活细胞层中分布着的树突状细胞，在触发免疫反应中起至关重要的作用。由于在分布着高密度的树突状细胞的皮肤活细胞层中，痛觉神经的分布很少，透皮免疫可以实现有效和无痛的疫苗接种。但是，实现有效透皮免疫的最大挑战在于输送足够剂量的抗原（疫苗的有效成份）透过皮肤的角质层屏障，到达树突状细胞。本研究中，我们使用微针阵列和纳米颗粒载体两种方法来增加抗原的透皮输送。我们使用的是实心微针阵列，其中的微针长度小于 1 毫米，直径小于 300 微米。用于在施用疫苗前对皮肤进行预处理。我们首先使用微针阵列对小鼠进行的白喉和流感疫苗的透皮免疫实验。在此基础上，使用不同的免疫佐剂，比如 *lpxL1* 脂多糖，皂甙 QuilA，CpG 寡聚核苷酸和霍乱毒素，与白喉抗原混合涂布在微针阵列处理后的皮肤上，研究它们对免疫反应的调节作用。另一种增加疫苗透皮输送的途径是使用柔性纳米颗粒载体。这种剂型包含由两性表面活性剂的双分子层组成的油包水颗粒结构。根据以往的研究，纳米颗粒的柔性以及促进小分子药物穿透皮肤的性能取决于它们的组成。我们发展了两类不同的抗原-纳米颗粒剂型并对它们的胶体性质进行了测定。通过将它们涂布到完整的和经过微针处理的小鼠皮肤上，对它们增强免疫反应的能力进行了研究。我们也添加了免疫佐剂以增加疫苗剂型的免疫原性。

具体来说，改进透皮免疫效果的努力开始于微针阵列的使用，在**第三章**中，我们使用了一个经过优化的微针阵列投射装置，该装置可以使短至 300 微米的微针阵列均一和可重复的刺透小鼠皮肤的角质层。穿刺的效果通过苔酚蓝染色和透皮水分散失的测量显示。使用这一组合，我们进行了白喉和流感抗原的透皮免疫实验，并使用霍乱毒素作为免疫佐剂。实验表明，对于白喉疫苗，使用微针阵列预处理皮肤对于诱导出显著的免疫球蛋白和中和抗体滴度起到了关键的作用，可以使抗体滴度较完整皮肤增加 1000 倍。添加霍乱毒素进一步增强免疫反应，使抗体滴度达到和传统疫苗接种方法（皮下注射白喉铝佐剂疫苗）相近的水平。与此形成鲜明对比的是：微针阵列对皮肤的预处理对增进流感抗原

的免疫保护并没有显著的效果。但是霍乱毒素的添加却可显著增强其免疫原性。这一结果显示；在透皮免疫中，微针阵列对皮肤的预处理对免疫效果的作用依赖于抗原本身的属性。也因此，后续的实验只使用白喉类毒素作为模型抗原。

由于经皮疫苗接种和经传统注射方式将疫苗输送至不同亚型的树突状细胞，免疫佐剂的作用也可能有所不同。在**第四章**，我们研究了不同免疫佐剂对于经皮输送抗原的免疫调节作用。我们已知微针阵列对皮肤预处理可显著增强白喉疫苗的免疫反应。添加不同的佐剂不同程度的提高了白喉特异的免疫球蛋白滴度：其效果排序为：皂甙 QuilA<CpG 寡聚核苷酸<霍乱毒素。而 IgG1/IgG2a 两种亚型免疫球蛋白的比值按以下顺序排列：无佐剂<皂甙 QuilA<霍乱毒素<CpG 寡聚核苷酸，说明在这一疫苗输送途径中，取决于使用佐剂的种类，免疫反应的类型可以从无佐剂时的 Th2 偏向型调节到 Th1 偏向型。这一研究表明，经皮疫苗输送诱导的免疫反应也可以通过使用免疫佐剂来进行调节和优化。

据报道，柔性纳米颗粒可以有效的促进小分子药物的透皮吸收，在**第五章**中，我们设计和发展了一种基于表面活性剂的白喉疫苗纳米颗粒剂型。这种纳米颗粒载体由蔗糖-月桂酸脂和磺化琥珀酸钠盐组成，八溴乙烯月桂酸脂被添加以增加脂双层的柔性。研究的参数包括：组成成分的摩尔比，白喉类毒素的浓度，缓冲液的种类，pH 值以及离子强度。疫苗的剂型根据胶体稳定性和白喉类毒素与颗粒的结合比例进行了优化。我们发现系统的 pH 值对白喉类毒素和纳米颗粒的相互作用有显著的影响。pH 值 4.5 的时候，超过 70%的抗原和纳米颗粒结合，而在 pH5.0 的时候，只有约 20%和纳米颗粒结合。同时，疏水相互作用在这个过程中起到重要的作用，白喉类毒素的蛋白结构完整性在制备过程中也得到了保留。

在**第六章**的研究中，我们综合使用了上述两种方式。在完整的或者经过微针阵列预处理过的小鼠皮肤上涂布了在第五章中发展的带负电的纳米颗粒白喉疫苗剂型，并且采用的开放式和封闭式两种涂布方式。然而，和单独使用白喉抗原溶液相比，纳米颗粒剂型并没有显著提高抗原的免疫原性。根据后续实验的结果，我们推测，纳米微粒在加载白喉抗原后柔性的丧失、它的带负电性、以及皮肤的中性 pH 环境造成的白喉抗原从纳米颗粒上的迅速释放，可能是造成这种结果的原因。根据这样的推测，我们发展了在中性 pH 环境下和白喉抗原稳定结合的阳离子脂质体剂型。其中的脂质体由大豆卵磷脂、DOTAP（一种带正电的两性分子）通过高压挤出法制备。制得的载有白喉抗原的脂质体直径约 150 纳米，性质稳定。和上述的带负电纳米颗粒剂型一样，我们测定了这种剂型的

物理化学和胶体属性，如 ζ-表面电位，抗原结合率以及颗粒的柔性。在 pH7.4 时，绝大部分的白喉类毒素抗原和脂质体通过静电相互作用而结合。在剂型制备过程中加入 Span 80，可以在不改变白喉类毒素和脂质体结合比例的情况下，使脂质体保持柔性。在接下来进行的透皮疫苗接种实验中，我们尝试了开放式和封闭式涂布于完整皮肤表面，以及封闭式涂布于经微针预处理的皮肤表面。遗憾的是，在这些情况下，这种带正电的白喉类毒素-脂质体剂型没有显示出较白喉类毒素抗原本身更强的免疫原性。通过与皮间注射对照组的比较，我们发现白喉类毒素的刚性脂质体颗粒剂型较类毒素抗原更难透过皮肤上经微针处理产生的微小孔道。此外，在体外的细胞培养实验中，带正电和带负电的这两种纳米颗粒和树突状细胞培养基混合时，对于树突状细胞的成熟没有明显的刺激作用。

综上所述，在完整皮肤表面涂布白喉类毒素，流感抗原，或者结合白喉类毒素的纳米颗粒剂型都不能引发有效的免疫保护。流感疫苗的透皮接种效果可以通过添加佐剂显著增强，但不受微针阵列对皮肤预处理的影响。而白喉抗原的透皮免疫可以通过微针阵列预处理皮肤，以及和免疫佐剂的共同施用来增强免疫效果，获得有效的免疫保护，但所研究的纳米颗粒剂型对此没有帮助。

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Zhi Ding in Leiden

Curriculum Vitae

Zhi Ding was born on June 15th, 1977 in Suqian, Jiangsu, China. In 1994 he completed his high-school education with specialization in mathematics and natural science at Suqian High School, Jiangsu, China. At the same year, he started his bachelor study at the Department of Biochemistry, Nanjing University, China. He conducted research on the project entitled "*Quantitative Modeling of Eukaryotic Gene Transcription Control"* for his undergraduate dissertation in the group of Prof. Dr. Wang at the State Key Laboratory of Pharmaceutical Biotechnology, Department of Biochemistry, Nanjing University, China. After his graduation in 1998, he stayed in Nanjing University, working as a study advisor and teaching assistant for undergraduate students in the same department. In the year 2000, apart from his administrative job, he started his graduate study, exempted from the entrance examination. He carried out the research project *Surface Modification of Biomaterial for Tissue Engineering* under the supervision of Prof. Dr. Zhang in the State Key Laboratory of Pharmaceutical Biotechnology, Department of Biochemistry, Nanjing University, China. In August 2004, he came to the Netherlands, where he started his study entitled *Master of Philosophy* for one year, which was continued as a PhD project in the Division of Drug Delivery Technology, Leiden University. Under the supervision of Prof. Dr. J.A. Bouwstra, Prof. Dr. W. Jiskoot and Dr G.F. Kersten he worked on *the subject Transcutaneous Immunization of Diphtheria Toxoid Using Vesicular Carriers and Microneedle Arrays*.

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