Mechanistic studies on transcutaneous vaccine delivery : microneedles, nanoparticles and adjuvants

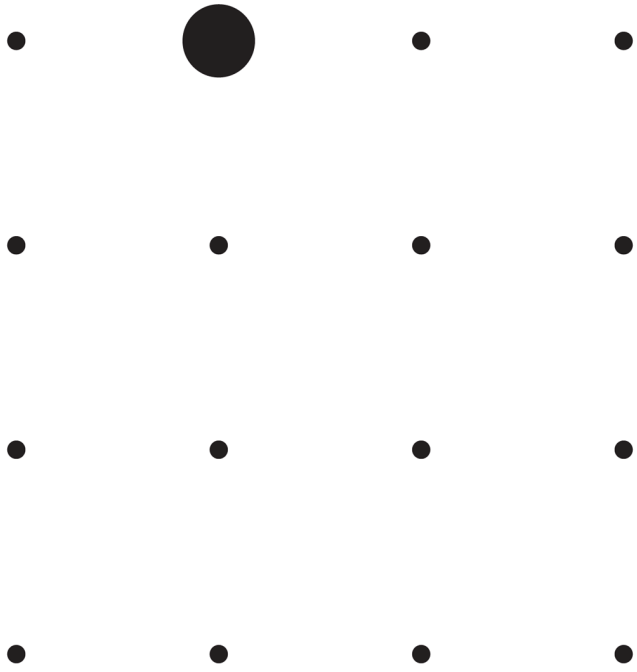
Bal, S.M.

Chapter 2

Advances in transcutaneous vaccine delivery: do all ways lead to Rome?

Suzanne M. Bal*, Zhi Ding*, Elly van Riet, Wim Jiskoot, Joke A. Bouwstra

* Authors contributed equally



Journal of Controlled Release 2010, 148(3): 266-282

Abstract

Transcutaneous immunisation (TCI) is a promising alternative to vaccine delivery via the subcutaneous and intramuscular routes, due to the unique immunological characteristics of the skin. The increasing knowledge of the skin immune system and the novel delivery methods that have become available have boosted research on new vaccination strategies. However, TCI has not yet been exploited to its full potential, because the barrier function of the stratum corneum, the top layer of the skin, is difficult to overcome. In this review we first discuss the immune system of the skin, focusing on the role the different types of skin residing dendritic cells play in the immune response. Subsequently, adjuvants and the large variety of devices, in particular microneedles, developed to deliver vaccines into the skin are summarised. Clearly, many ways have been explored to achieve efficient transcutaneous vaccination with varying success. The perspectives of the most promising concepts will be discussed.

Introduction

Over the last two centuries, vaccination has been one of the most successful medical interventions in reduction of infectious diseases [1, 2]. A good vaccine is safe, administered in a minimally invasive manner and, most importantly, capable of eliciting a strong, protective immune response. Currently available vaccines can be classified into three categories: live-attenuated, inactivated and subunit vaccines. From a safety perspective subunit vaccines are preferred over live-attenuated and inactivated pathogens. However, purified antigens generally are poorly immunogenic and therefore require to be formulated with adjuvants [3, 4].

Nearly all subunit vaccines are administered by intramuscular (IM) or subcutaneous (SC) injection, but alternative routes of administration are widely explored in the search for more effective and safer vaccines. Injection requires syringes, needles, and trained personnel. Moreover, injection can be painful and cause stress, especially in children. For pediatric vaccination programs, poor compliance is one of the reasons for incomplete vaccination coverage [5]. Finally, muscle and SC tissue contain less antigen presenting cells (APCs) than skin tissue, adding to the belief that they are not ideal sites for vaccination. The disadvantages of injectable vaccines have boosted the research on nasal [6], transcutaneous [7], oral [8] and pulmonary delivery of vaccines [9].

The transcutaneous route is particularly attractive because the skin is highly accessible and has unique immunological characteristics. It has been known for a long time that an effective immune response can be induced via the skin and many different approaches have been tried. One successful example of transcutaneous vaccination is scarification in the case of smallpox immunisation in humans [10]. The presence of professional antigen presenting cells (APCs) in the epidermis and dermis mediates the immune response following cutaneous immunisation [11]. Another primary reason for considering the transcutaneous route is the potential for safe immune stimulation, as it avoids the direct contact between potent (sometimes even slightly toxic) adjuvants with the general circulation [12]. However, the uppermost layer of the skin, the stratum corneum, acts as a barrier for diffusion and thereby forms a major obstacle to transcutaneous immunisation (TCI), e.g. vaccination through intact or pre-treated skin. Currently, the main challenges for cutaneous immunisation are to enhance the transport of antigens across the skin barrier and to improve the immunogenicity of topically applied subunit vaccines.

This review will focus on approaches for improving TCI. It starts with a description of the barrier and immunological functions of the skin. As TCI is an emerging field, many techniques have been employed to elicit an efficient immune response. We will summarize these techniques and make a distinction between approaches for enhancing transcutaneous antigen delivery and for improving the immunogenicity of subunit vaccine

formulations (addition of adjuvants). For clarity, different terms related to immunisation via the skin are defined in Table 1.

Table 1. Skin immunisation.

Term	Interpretation
Cutaneous immunisation	Both intradermal and transcutaneous immunisation
Intradermal immunisation	Antigen delivery into the dermis via a syringe and hollow needle
Transcutaneous immunisation	Antigen delivery into the epidermis and/or dermis through intact or pre-treated skin

Immunological function of the skin

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 (figure 1). The epidermis is the outermost layer of the skin. The human epidermis varies in thickness from 50 to 150 µm. The barrier function of the skin is located in the upper 15-20 µm, 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 results in a practically impermeable barrier which reduces the passage of molecules, especially those larger than 500 Da [13]. Underneath the stratum corneum resides the viable epidermis. The main cell type in the viable epidermis is the keratinocyte. However, melanocytes, Merkel cells and Langerhans cells (LCs, figure 2), although less abundantly present, also play important roles in the functioning of the viable epidermis. Underneath the viable epidermis the dermis is located. The important cell classes in the dermis are fibroblasts, mast cells, and dermal DCs (dDCs). The dermis also contains blood vessels, lymph vessels, nerves and an abundant level of collagen fibres. This skin layer is the major site of cellular and fluid exchanges between the skin and the blood and lymphatic networks [14]. Beneath the dermis lays the subcutaneous fat tissue, an assembly of adipocytes linked by collagen fibres. It forms a thermal barrier, but also stores energy and functions as a mechanical cushion for the body [15]. 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 in the skin structure [14].

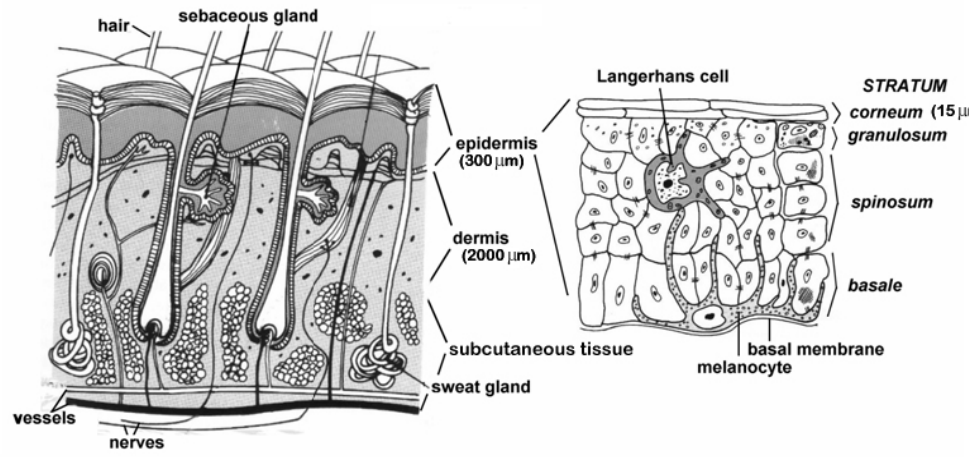


Figure 1. Structure of the skin. The skin consists of three main layers: epidermis, dermis, and subcutaneous fat tissue. The barrier function of the skin is located in the uppermost layer, the stratum corneum. Image adapted from Watt [16].

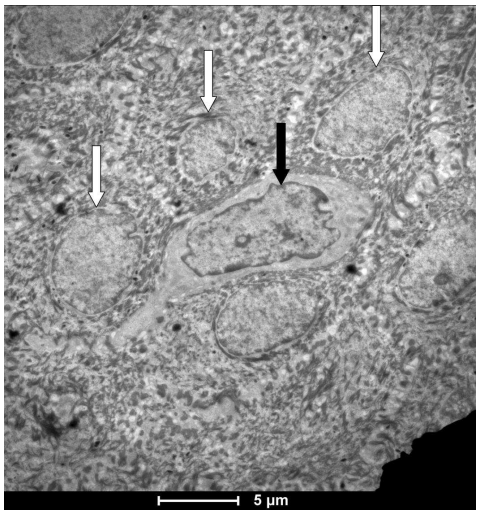


Figure *2.* Electron microscopy image of human skin, showing keratinocytes (white arrows) and a LC (black arrow).

Besides the barrier function, the skin also has important immunological functions with an imperative role for the skin residing APCs, such as LCs and dDCs, which communicate with keratinocytes, mast cells and subsets of T lymphocytes. 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 questions: whether or not to respond, and how to respond. This decision can be a matter of life and death exemplified by for instance leprosy [17]. The skin is involved in both innate and adaptive immunity. The adaptive response can generate memory responses and therefore generally becomes more effective with each successive encounter with the same antigen, whereas the innate immune mechanism provides an immediate, but short-lasting defence against infections. The immune system of man and mouse differ in several aspects; unless stated otherwise, in this review the human immune system is discussed.

Innate immunity

The innate immune system fights infections in an unspecific manner using fever, the complement system, phagocytic and natural killer cells, naturally occurring antibodies and anti-microbial peptides (figure 3).
Keratinocytes, accounting for about 90% of the total epidermal cell population, play an important role in innate immunity in the skin. In case of danger, *e.g.* skin barrier disruption, keratinocytes produce a wide range of cytokines, chemokines and antimicrobial peptides [18]. In this way they are able to kill invading pathogens and recruit immune cells. Examples are the cytokines interleukin-1α (IL-1α), IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α), which interact with DCs and help to maintain an appropriate balance between reactivity and tolerance of the immune system [19, 20]. For example, migration and maturation of LCs are initiated by pro-inflammatory cytokine IL-1β and keratinocyte-derived TNF-α [21, 22]. Another example is the expression of CCL20 by keratinocytes that attracts LCs [23, 24]. In addition keratinocytes have been reported to function as non-professional APCs, via surface expression of major histocompatibility complex (MHC) class II molecules [25].
Besides keratinocytes, also neutrophils, macrophages, mast cells and natural killer cells secrete cytokines that influence DC maturation [26-28]. DCs are the most important APC in the skin and play a vital role in both the innate and adaptive immune response. Skin residing DCs, LCs and dDCs, 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 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 [29]. Among agents that trigger these receptors pathogen-associated molecular patterns (PAMPs) are most relevant in the context of this review. PAMPs usually represent exogenous signals, such as the conservative motifs of microbial products [30]. The function of DCs in the initiation and regulation of the adaptive immune response will be discussed later in this review.

Pattern-recognition receptors

The innate immune response is mediated by the PRRs, of which Toll-like receptors (TLRs) have been a central focus for immunologists and vaccinologists after they were discovered by Medzitov and Janeway in 1997 [31]. TLRs are important PRRs involved in host defence against a variety of pathogens in general and also in the skin. So far, ten TLR members have been identified in humans and three more in mice, each thought to selectively recognize

diverse bacterial or viral stimuli or endogenous signals [32]. TLRs can be divided into subfamilies, according to the ligands they recognise and to their cellular localisation. The subfamily of TLR1, 2, 4 and 6 recognises lipids, whereas TLR3, 7, 8, and 9 recognise nucleic acids [30]. Generally, TLRs detecting bacterial products other than nucleic acids (TLR1, 2, 4, 5, 6 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 [33].
In the skin, most studies focus on TLR expression on LCs and dDCs, which is dissimilar and also differs from other subtypes of DCs at mucosal surfaces or in the blood circulation. Epidermal LCs freshly isolated from the human skin express TLR1-3, 6 and 10 but not TLR4 and 5 [34, 35]. dDCs do express TLR4 and 5, in addition to TLR2, 6, 8 and 10 necessary for recognition of bacterial PAMPs [36]. Besides DCs, keratinocytes also express TLR1-6, 9 and 10 [37-40]. Furthermore, Yu et al. recently showed that cultured human melanocytes express TLR2-4, 7 and 9 [41], which attributes a possible role for these cells in the immune response. The TLR distribution on immune active skin cells (human and mouse) are presented in Table 2. 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.

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, 6, 8, 10	9
Myeloid DCs	1-4	1-4, 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

Table 2. TLR distribution in immune active skin cells [34-40, 42-48].

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. It is generally accepted that the detection of pathogens by TLRs initiates the mobilization of the host defence 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 [49]. In the absence of TLR activities, most viruses and intracellular bacteria are recognized by alternative intracellular receptor families, including nucleotide oligomerisation domain (NOD)-like receptors (NLRs) [50-52], retinoic acid inducible gene based (RIG)-I-like receptors (RLRs) and C-type lectin-like receptors (CLRs) [53-55]. In general, activation and maturation of DCs are the consequence of signal

transduction within the PRR network, resulting in appropriate immunity against invading pathogens.

Adaptive immunity

Adaptive immunity provides pathogen-specific, long-lasting protection to the host. Similar to those at other immunological sites, skin DCs are an important link between innate and adaptive immunity (figure 3) [29]. Upon activation, the DCs will maturate and migrate to the lymph nodes, where they present epitopes via MHC I and II to respectively $CD8^+$ and $CD4^+$ T cells [29]. Adaptive immunity starts with activation and polarization of lymphocytes via DC-T cell interaction, followed by proliferation of T and B lymphocytes in the secondary lymphoid organs (figure 3). T and B 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 each B- or T-cell receptor specifically recognizes a unique epitope. Below we will briefly describe the general function of T and B cells in the immune response, followed by the specific role of skin DCs in induction of the adaptive immune response.

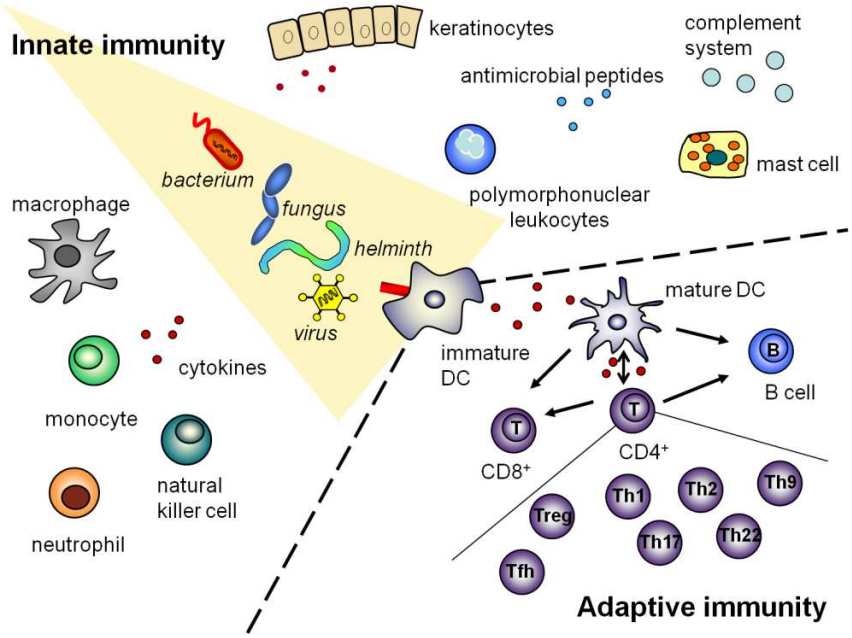


Figure 3. Schematic representation of the cells involved in the general innate and adaptive immune response. Upon infection with a pathogen the cells of the innate immune response offer immediate, but short-lasting help. This leads to DC activation, which forms the bridge between the innate and adaptive immunity. The cells of the adaptive immune response provide pathogen-specific, long-lasting protection.

Effector cells of the adaptive immune response

T cell activation depends not solely on specific recognition by the T cell receptor of antigen presented by APCs; the interaction of co-stimulatory molecules (CD80 and CD86 on APC with CD28 on T cells), the secretion of stimulatory cytokines (IL-2) and a polarization signal (*e.g.* IL-4 and interferon-γ (IFN-γ)) are also necessary [43, 56]. TLR recognition by APCs contributes to this activation process. As mentioned above there are two different types of effector T lymphocytes, but the $CD4^+$ T cells (also called T helper, or Th cells) are further classified in different subsets. The best studied subtypes are the Th1 and Th2 cells. Most bacterial and viral products, including nearly all TLR ligands drive the differentiation towards a Th1 functional phenotype [57, 58]. Th1 cells secrete IL-2 and IFN-γ cytokines, support the production of IgG2a antibodies in mice (IgG1 in humans) and stimulate cell-mediated immunity against intracellular pathogens [59]. In the presence of parasitic pathogens and allergens, naïve $CD4^+$ T cells differentiate into Th2 cells. Th2 type cytokines, including IL-4, IL-5 and IL-13, mediate humoral immunity and support the production of the IgG1 (in mice) and IgE subclasses. The discovery of Th17 that are induced by extracellular bacteria and were also implicated to have a role in (auto)immune disorders, regulatory T cells (Treg), follicular helper T cells (Tfh) [60-62] and more recently also Th9 and Th22 [63-66], the latter being described to be important in skin homeostasis and pathology [65, 66], further complicates the $CD4^+$ paradigm (figure 3). The dominant type of immune response induced is determined by many factors, including the route of antigen delivery, antigen dose, duration of antigen presentation, number, or frequency of immunisations and inclusion of adjuvants. The main function of $CD8^+$ T cells is to kill tumour cells or cells infected by viruses or intracellular bacteria. Naïve $CD8^+$ T cells become cytotoxic T cells (CTL) 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 to clear infected or tumour cells. The activation of a $CD8^+$ T cell response is the main mechanism of vaccines developed for cancer therapy. Th1 $CD4^+$ T cells seem to be required to help $CD8^+$ T cells fight certain pathogens. Cross-talk between both types of effector T cells is mediated by CD40-CD40L interactions [67].

The humoral immune response is mediated by B lymphocytes. These cells recognize 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 activated $CD4^+$ T cells. 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 [68]. B cells can take up antigens and present them by MHC II to $CD4^+$ T cells. Interactions between B cells and $CD4^+$ T cells mutually stimulate each other. Activated Th2 cells express

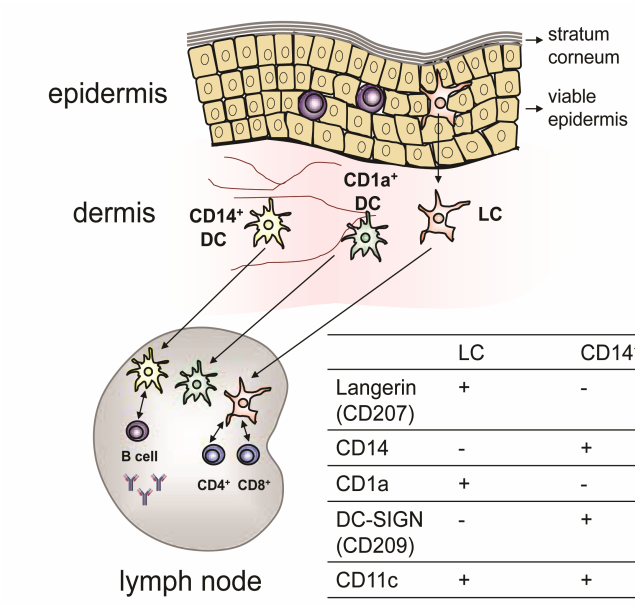
CD40L on their surface which can interact with CD40 on B cells. In this way, the activation of more effector T cells and the production of antibodies are sustained [69]. These antibodies assist in the destruction of microbes by binding to them, thereby making them easier targets for phagocytes and facilitating activation of the complement system.
The basis of vaccination lays in the existence of memory B and $CD4^+$ and/or $CD8^+$ T cells. These cells enable faster and stronger responses to pathogen-derived antigens encountered before [70]. They are long-lived and, upon contact with a familiar antigen, 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.
Interestingly, recent findings implicate an important role for skin resident T cells in memory responses. Not only were they found to outnumber the T cells in the blood [71], but in addition memory T cells were found to survive long-term in the skin and are crucial in the control of an infection upon a secondary challenge [72, 73]. Most interestingly for vaccination purposes, it was found that after antigen presentation to naïve T cells by DC in the lymph nodes, skin homing effector memory T cells were not only migrating to the site of infection, but distributed to all parts of the skin. After the pathogen was cleared, these cells remained resident locally in the skin. Moreover, during primary infection, proliferating T cells in the skin draining lymph nodes were also found to be distributed to lymph nodes draining other tissues, and subsequently these cells were found to reside in those peripheral tissues, including gut and lung [74]. How infection or immunisation via the skin can lead to local as well as systemic memory responses was recently reviewed by Clark [75] and implicates that immunisation through the skin can generate widespread systemic immunity through populations of tissue resident effector memory T cells.

Skin DCs as a bridge between innate and adaptive immunity

The DCs link the innate to the adaptive immune response. They not only sample the environment, but afterwards they process antigens and undergo a maturation and differentiation process. In the skin, differentiation of LCs and dDCs during maturation includes increased expression of MHC 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 migration of these cells from the skin to the draining lymph nodes [76, 77]. 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 [78, 79]. This occurs in an antigen-specific fashion and results in the T cell expansion into extremely potent immune stimulatory cells, controlling the development of adaptive immunity [80].

Several distinct types of DCs are present in human skin and this is an emerging field of research [81, 82]. The most evident distinction is between the LCs and the dDCs, 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 [83]. Human LCs can be distinguished from other subsets of DCs by their expression of langerin/CD207, a C-type lectin that induces the formation of a unique intracytoplasmic organelle, the Birbeck granule [84]. Furthermore LCs express E-cadherin and high levels of CD1a, responsible for the presentation of lipid antigens to T cells [85]. Two subsets of DCs in the dermis have been distinguished until now: $CD14^+$ dDCs and $CD1a^+$ dDCs [86]. $CD14^+$ dDCs are most easily characterized by expression of DC-SIGN (DC-specific intercellular adhesion molecule-3 (ICAM-3)- grabbing non-integrin), also known as CD209, in addition to CD1c and CD11b [87, 88]. Dermal $CD1a^+$ DCs were shown to express an intermediate phenotype between $CD14^+$ DCs and LCs (figure 4, [36]). dDCs 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 mice, i.e. the langerin$^+$ CD103$^+$ dDC [89-91]. This subtype differs from LCs and the classical dDCs by a low expression of CD11b and high expression of CD103 [92, 93]. Furthermore, LCs were found to express epithelial-cell adhesion molecule (EpCAM) [91, 94], an adhesion molecule that distinguishes them from both types of mouse dDCs. There is some speculation that the $CD1a^+$ subset of dDCs in human skin might correspond to the langerin$^+$ CD103$^+$ dDC found in mice, but that still remains to be investigated [81].



	LC	CD14$^+$ DC	CD1a$^+$ DC
Langerin (CD207)	+	-	-
CD14	-	+	-
CD1a	+	-	+
DC-SIGN (CD209)	-	+	-
CD11c	+	+	+
CD11b	-	+	+

Figure 4. Dendritic cells present in the epidermis and dermis of human skin. These cells differ in respect to the expression of cell markers and the interaction with cells of the adaptive immune response. While LCs are more involved in the interaction with $CD4^+$ (to preferentially induce Th2) and $CD8^+$ T cells, $CD14^+$ dDCs have the ability to induce B cells to switch isotype and become plasma cells by direct contact and via the induction of follicular helper T cells ($CD4^+$). $CD1a^+$ dDCs express an intermediate phenotype [88, 95-98].

The different DC subsets in human skin each have distinct functions in the adaptive immune response (figure 4). Both dDCs and LCs isolated from human skin were shown to activate naïve $CD4^+$ T cells, but LCs induced the secretion of Th2 type cytokines, which $CD14^+$ DCs did not [95]. The $CD1a^+$ dDCs provoked some secretion of Th2 type cytokines, but less compared to the LCs. $CD14^+$ dDCs promote the differentiation of naïve B cells into IgM-secreting plasma cells through the secretion of IL-6 and IL-12 [99, 100]. This effect was not observed with LCs, which not only failed to induce high levels of IgM production, but isotype switching of naïve B cells and the production of IgG was also only induced by $CD14^+$ dDCs [95]. These results, together with studies performed in mice showing that $langerin^-$ DCs preferentially migrated to the outer paracortex of the lymph nodes, just beneath the B cell follicles [101, 102], indicate that $CD14^+$ dDCs are important for the induction of humoral immune responses.

Human LCs were shown to have a function in the $CD8^+$ T cell response [95]. Both isolated and *in vitro* cultured LCs were shown to induce proliferation of naïve $CD8^+$ T cells to a higher extent than $CD14^+$ dDCs [95, 103]. Also in mice a role was ascribed to LCs in the cross-presentation of antigens to $CD8^+$ T cells, as it was shown that upon stimulation $langerin^+$ DCs migrate into the T cell-rich inner paracortex [102]. However, in this study the relative contribution of LCs and $CD103^+$ $langerin^+$ was not explored. This would be very interesting as a recent study suggests that in mice the $CD103^+$ $langerin^+$ dDCs are responsible for cross-presentation *in vivo* [92]. Coming back to the immune response in humans, the function of the $CD1a^+$ dDC remains not fully understood. Currently, this topic is of great interest and important for the design of novel vaccines targeting specific DC subsets [104].

Even though these studies clearly indicate the crucial role of skin resident DCs in the immune response, soluble antigen can also directly diffuse to the draining lymph nodes through the lymphatic system [105]. Here the antigen can be taken up by a large population of lymph node resident DCs. This process is much faster, thereby inducing two distinct waves of antigen delivery to lymph nodes [101], which can induce different immune responses [106].

Transcutaneous immunisation

As mentioned before, for subunit vaccines the co-application of adjuvants with the antigen(s) is required for induction of a strong immune response. This approach also holds for TCI, but for successful TCI transport of the antigen and adjuvant across the skin poses an additional challenge. Here we will briefly discuss the adjuvants used for TCI, followed by a more in depth overview of the physical methods utilized to overcome the stratum barrier.

Adjuvants used in TCI

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-Welder *et al.* [4]). The most commonly used adjuvants are colloidal aluminium hydroxide and aluminium phosphate, commonly referred to as alum [107, 108]. Other adjuvants recently approved for human use are monophosphoryl lipid A (MPL) and MF59, an oil-in water emulsion containing squalene, which has been accepted in Europe. These three adjuvants have not been used in TCI, probably due to their relatively large size, which limits transport across the skin barrier. Furthermore the depot effect of alum is undesirable for TCI. There are many other experimental adjuvants commonly used; here we will focus on those used for TCI. Since these adjuvants are still in pre-clinical development, the discussion below concerns animal (mouse) studies.

Bacterial exotoxins

Bacterial ADP-ribosylating exotoxins possess a high degree of adjuvanticity and are therefore the adjuvants that are most often used pre-clinically for TCI. Among them, cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) are the ones most intensively studied [109]. CT and LT bind to the GM1-ganglioside receptor (B subunit) and have ADP-ribosyl transferase activity (A subunit) [110-114]. CT and LT do not only function as adjuvants, but in addition provoke the formation of anti-CT and -LT antibodies. In the first TCI study, Glenn *et al.* showed that application of CT on intact mouse skin resulted in anti-CT antibodies [115]. As this was an excellent result, this study was followed by many others showing that CT enhances the immune response against other antigens [115-120]. LT was shown to possess similar adjuvanticity [118, 121-125]. These studies are summarized in Table 3A. CT and LT do not only improve the total immune response, but affect the quality of the immune response as well, although this is still under debate. While there are studies indicating that mainly a Th1 bias with enhanced IgG2a levels is induced [126-130], others point to a Th2 bias [119, 120, 123, 131] or a mixed response [121, 122]. Besides antibody responses, it was shown that CT can induce a cytotoxic T cell response [120] and that the CTA and CTB subunit are responsible for different cytokine expression from restimulated lymphocytes isolated from the spleen of immunized mice [132]. Of course the antigen, mouse model and dose can also have a profound influence on the elicited immune response. Additional studies are needed to further elucidate how CT and LT affect the immune response. It remains an important question how bulky antigens as well as adjuvants can penetrate the stratum corneum barrier when applied on intact skin. Beignon

et al. showed that CT could penetrate hydrated mouse skin *in vivo*, and was found preferably around the hair follicles [133]. However, there are also studies in which the skin is pre-treated by abrasion, which could play a significant role.

TLR ligands

As described above, TLRs are important signalling molecules which cells use to sense danger. It is therefore a logical approach to use either purified or synthetic TLR ligands as adjuvants for vaccination purposes. One example is CpG. Prokaryotic DNA contains unmethylated CpG dinucleotides within nucleic acid motifs that are recognized by TLR9 of vertebrates [134]. By signalling through TLR9, CpG induces the secretion of pro-inflammatory cytokines such as IL-12, TNF-α and IFN-γ, resulting in a Th1 biased response [135, 136]. Scharton-Kersten et al. first showed that CpG functions as an adjuvant when applied with DT on intact skin, as elevated anti-DT IgG titres were observed [121]. The same was observed for TCI with CpG co-administered with the model antigen ovalbumin (OVA) or DNA vaccine encoding influenza M protein [131, 137]. In the skin CpG induces LC and DC maturation and migration of these APCs to the lymph nodes [138, 139]. CpG is capable of modulating pre-existing immune response causing a switch from a Th2 biased response to a Th1 biased response [131, 140, 141]. Topical application of a HIV peptide together with a mixture of CT and CpG induced a strong HIV-specific CTL response resulting in protection against a mucosal challenge [142]. CpG has been used as an adjuvant in clinical immunisation studies using different vaccination routes [143-145], however not yet in clinical TCI studies. Other TLR ligands have also been used pre-clinically in TCI studies and are summarised in Table 3A. The many different strains and type of vaccines used in influenza immunisation studies are listed in Table 3B.

Table 3B. Type of influenza antigen used in studies mentioned in Table 3A.

Reference	Type of influenza vaccine used
[118, 131, 147]	HA:307-319 peptide
[137]	M protein DNA vaccine
[127]	H3N2 subunit vaccine (A/Panama/2007/99 RESVIR-17)
[129]	Inactivated H1N1 virus (A/PR/8/34)

Physical methods to overcome the skin barrier

Disruption of the skin barrier increases the transcutaneous permeation of antigen and makes it more readily available for sampling by APCs (figure 5). Moreover, it is known that skin barrier disruption can activate the immune system, inducing the secretion of pro-inflammatory cytokines by keratinocytes and resulting in DC activation [152, 153]. This makes it attractive to develop physical methods to overcome the skin barrier.

Table 3A. Adjuvants used in pre-clinical TCI studies.

Adjuvant	Dose (µg)	Antigen	Dose (µg)	Type of immune response	Reference
CT	100	DT	100	IgG, mixed IgG1/IgG2a, $CD4^+$	[115, 117, 121, 146]
	100	DT	100	IgG, IgG2a	[126, 127, 130]
	25-100	TT*	25-50	IgG, $CD4^+$	[115, 122]
	25-50	Influenza	100	IgG, IgG1, $CD4^+$	[118, 131, 137]
	100	Influenza	30-100	IgG, mixed IgG1/IgG2a	[127, 129]
	100	VP1 (FMD**)	100	IgG1	[133]
	100	G2Na/G5 (RSV***)	150	IgG1	[119]
	50-100	OVA	25-300	IgG, IgG2a, CTL, $CD4^+$	[120, 132]
	50	PCLUS3-P18IIIB (HIV****)	50	CTL	[142]
LT (and derivatives)	20-100	DT	10-100	IgG, IgG2a, $CD4^+$	[121, 128]
	1-100	TT	5-100	IgG, IgG1, $CD4^+$	[121-123, 147]
	100	β-gal	100	IgG	[118]
	50	BSA*****	100	IgG	[125]
	50	Influenza	100	$CD4^+$	[118, 147]
CpG	10-100	DT	5-100	IgG, IgG2a, $CD4^+$	[121, 140]
	12.5-100	Influenza	100	IgG2a, $CD4^+$	[131, 137]
	100	TT	20	IgG, IgG2a	[123]
	50	HIV	50	CTL	[142]
	50-500	OVA	50-100	IgG, CTL, $CD4^+$	[148, 149]
TLR7 Imiquimod Resiquimod	50000	OVA	150	CTL	[150]
	100	OVA	100	CTL	[151]

* TT: tetanus toxoid
** FMD: feet and mouth disease
*** RSV: respiratory syncytial virus
**** HIV: human immunodeficiency virus
***** BSA: bovine serum albumin

One important factor to consider is what/where to target. As mentioned above LCs and dDCs in the skin are located in different skin layers and have a dissimilar, but not yet completely understood, function in the skin immune system. Therefore, to assume that any means of barrier disruption will lead to the desired immune response is not true. Development of vaccine delivery devices should go in close collaboration with immunological studies into the exact function of the skin residing immune cells. The clinical safety of the devices described here, though of major importance, has received little attention so far. As the scope of this review is on the efficacy rather than the safety and the latter was recently reviewed by Donnelly et al. [154], we will not discuss this subject. The most widely used method to date to overcome the skin barrier for cutaneous immunisation is intradermal (ID) injection, invented by Mendel and Mantoux in the early 1900s [155] (figue 6A). With ID injection it is possible to deliver antigens into the dermis precisely and reproducibly. Clinical trials with hepatitis B, influenza, and therapeutic cancer vaccines have shown that ID vaccination is safe and effective. In many cases, stronger immune responses with a lower antigen dose compared to SC or IM injection were observed [156, 157]. However, traditional ID injection requires well-trained healthcare workers; therefore new devices for ID injection are being developed. One example is the Becton Dickinson (BD) microinjection system, Soluvia™ (figure 6B). 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) [158, 159]. These studies underline the effectiveness of the skin as a site of immunisation, but ID injection still employs long needles and causes pain. For vaccination of healthy people TCI in a minimal-invasive manner would be more desirable.

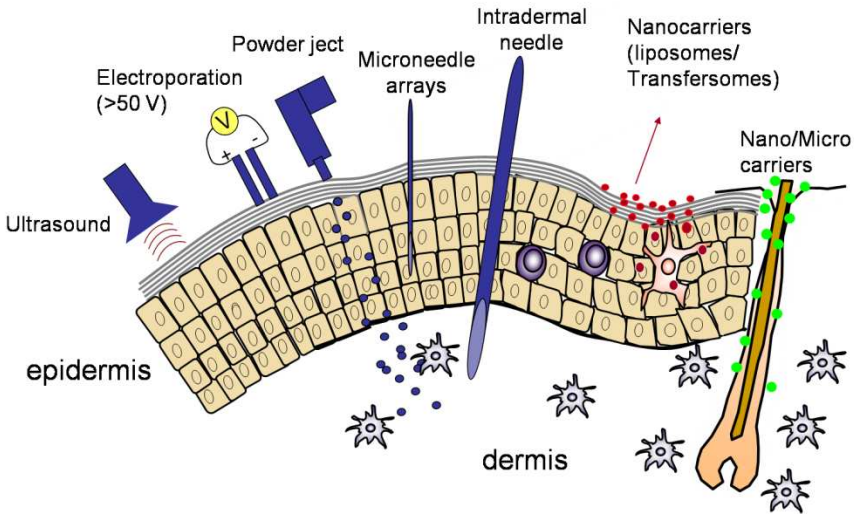


***Figure* 5**. Schematic illustration of several physical approaches and devices developed for TCI.

Microneedle arrays

One approach towards painless TCI is to dramatically reduce the size of needles so that they are barely perceptible. The concept of the microneedle array for drug delivery purposes essentially dates back to a patent, filed in 1976, by Gerstel and Place [160]. However, it was not until the 1990s that the technique became viable, as by then fabrication techniques became available to produce these microneedle arrays in a potentially cost-effective manner.

The term microneedles in the definition used here refers to needles shorter than 1 mm. Theoretically, microneedles only need to pierce the 15-20 µm thick stratum corneum before reaching the viable epidermis. However, the skin is an 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 [161, 162]. To ensure effective and reproducible piercing regardless of these factors, microneedles need to be much longer than 20 µm [163], although the use of an applicator may reduce the required microneedle length. Other parameters, such as microneedle diameter, insertion depth, microneedle tip geometry and microneedle density also influence skin perforation and antigen delivery [163-165]. For instance, very thin microneedles are fragile, which results in an increased risk for fracture in the skin. To overcome this risk, increased microneedle density helps to spread the surface forces between each microneedle, thereby decreasing the risk for fracture in the skin [166]. On the other hand, increased microneedle density can give rise to the 'bed of nails' effect and not improves antigen delivery [163].

Numerous methods have been developed to fabricate a wide range of microneedles as recently reviewed by Donnely *et al.* [154]. Microneedle technology is under active research and various strategies were developed using microneedle arrays in transdermal drug delivery, including TCI (Table 4A) [167, 168]. Below we will discuss the most important strategies pursued so far.

Solid microneedles

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 BSA through human epidermis *in vitro* after penetration with a microneedle array of 150 µm needle length (figure 6E) [169]. Banks *et al.* reported that the flux across microneedle array pre-treated skin was augmented by increasing the charge of the drug [170]. In our group, Verbaan *et al.* showed that 200 nm particles can diffuse through conduits formed by a solid microneedle array (300 µm long, 4×4 array, figure 6C) [171]. This microneedle array was applied at a speed of 3 m/s by an electric applicator. In the absence of such an

applicator no conduits were formed. For its application in TCI, Ding *et al.* have demonstrated that pre-treatment of the skin using the same type of microneedle arrays leads to a major improvement (1000-fold increase in antibody titres) in the immunogenicity of topically applied DT in mice [127]. The immune response was further boosted by co-application of CT. Given the fact that with microneedle pre-treatment only a fraction of the vaccine formulation applied is transported to the APCs in the skin, the dose of antigens can be further refined. We also showed that the immune responses induced can be additionally improved and modulated by selective addition of adjuvants [126], which may lower the antigen dose required. In general, microneedle array pre-treatment is considered a simple approach for TCI with great potential, but parameters such as dose and application time should be optimized. Recent studies indicate that the smaller the entity, the easier the transport through the conduits, thereby limiting the potential of for instance liposomes and nanoparticles as antigen carriers in TCI [130, 172]. More groups are currently focusing on using solid microneedles for skin pre-treatment [173, 174], and these systems could be used in future TCI studies. 3M has developed the Microstructured Transdermal System (MTS) using solid microneedles, either coated or uncoated [175]. In collaboration with VaxInnate these microneedles will be used for the delivery of an influenza vaccine.

Coated microneedle arrays

Arrays of vaccine coated microneedles have been developed as an alternative to microneedle pre-treatment. Coated microneedle arrays may not be very attractive for transdermal drug delivery as only a limited amount of active compounds can be coated onto the needles. However, this amount might be sufficient for antigens to generate a protective immune response [167]. The concept of coated microneedle arrays is that they are inserted into the skin and then removed, thus depositing their payload to a maximum depth determined by the length of the microneedle and the application manner. Matriano *et al.* showed, using 1 µg OVA on pre-coated microneedle arrays, a 100-fold increase in immune response compared to IM injection of the same dose [176]. They used an array with 300 µm long titanium microneedles, applied to the skin by an impact insertion applicator. Later, Widera *et al.* from the same group carried out an extensive study on the influence of OVA-coated microneedle properties on the immune response. 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) [163]. This led to the development of the Macroflux system® which is now in a phase I clinical study for TCI with an influenza vaccine (figure 6D).

Coatings are usually applied by dipping microneedles in a vaccine formulation. 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 antigen coated can be adjusted by the concentration of the coating solution. Coatings could be localized just to the needle shafts and formulated to dissolve within 20 s in porcine cadaver skin [177-180]. Another method is to use gas-jet coating, to achieve more uniform coating of densely packed microprojections (figure 6F) [181]. Two groups focusing on coated microneedles are the groups of Prausnitz and Kendall. While the former group uses rather long (up to 700 µm) and sparsely packed microneedles the latter uses very short (30-90 µm) and densely packed microneedles, also called Nanopatch™ [181-183]. Initially a large difference was reported in the amount of vaccine deposited in the skin, only 15% for the short densely packed compared to 90% for the long sparsely packed microneedles [178, 181]. However, by applying the short microneedles with a speed of 2.5 m/s their pay-load could be doubled, even though the majority of the vaccine still remained on the Nanopatch™ [183].

Both types of coated microneedles have been employed successfully for TCI, in immunisation studies with OVA, H3N2 influenza antigen, Fluvax® 2008, inactivated influenza virus and Hepatitis C DNA vaccine, with doses ranging between 0.4 and 10 µg [178-185]. Humoral and cellular antibody responses comparable to those induced after IM or gene gun immunisation were observed.

From a formulation point of view it could be an advantage that dried antigen formulated on the surface of the microneedles may improve the long-term stability [186]. However, coating of antigens has also been reported to reduce the immunogenicity of the vaccine, needing trehalose to partially retain the activity [185, 187].

Hollow microneedle arrays

By solid microneedle array pre-treatment, antigen delivery is based on passive diffusion along the conduits created by the microneedles. Although this is a relatively easy approach from a technical point of view, not all of the dose applied will be available to activate immune cells in the skin due to limited transport through the conduits. Using hollow microneedle arrays to inject the vaccine to a well defined depth in the skin, one can precisely steer the flow rate using a syringe or a pump and provide a more controlled vaccine delivery. The main technical demands are avoiding leakage and clogging of the microneedles during injection [188]. Clogging can be prevented by using a bevelled tip [164]. However, given the short needle length allowed, it will increase the chance for leakage. Therefore an optimum in the flow rate, needle length and localization of the opening are demanded. Furthermore, insertion of the microneedles using a drilling or

vibrating motion may avoid the tissue compaction [165, 189]. Martanto *et al.* investigated the influence of different parameters on the infusion flow rate and found the location of the tip opening and retraction of the microneedle before injection to be of major importance [164, 165].

The first hollow microneedle array, 150 µm long, made of silicon, was presented by McAllister et al. in the late 1990s [190]. Recently, the potential of hollow microneedles for vaccination purposes has received attention as it can both be used for TCI and ID vaccination depending on the microneedle length [7, 191]. Van Damme et al. delivered α-RIX influenza vaccine (3.3 µg of HA per strain) using a hollow microneedle array (450 µm long, 4×1, MicronJet® developed by Nanopass, figure 6H & 6I) and elicited immune responses similar to those induced by 15 µg HA per strain administered IM in human volunteers [156].

Dissolvable microneedle arrays

Usage of dissolvable or biodegradable materials containing the vaccine components is an elegant way to deliver a vaccine without the possibility of microneedles breaking off in the skin. Miyano *et al.* were the first to report about maltose based microneedles [198], followed by Ito *et al.*, who used dextrin microneedles for the delivery of insulin and erythropoietin [199, 200]. More data emerged recently, following this trend [201-205]. Before TCI studies using these systems, successful delivery of large molecules such as the above mentioned insulin and erythropoietin and also BSA [201, 203, 205] and IgG [204] was reported. Recently, Sullivan *et al.* showed that immunisation with polymeric dissolvable microneedles containing inactivated influenza virus resulted in a strong antibody and cellular response and provided protection against an influenza challenge [206].The main challenge is to develop a fabrication technique which allows antigen to be incorporated into the matrix of the microneedle materials in a mild procedure without causing antigen breakdown and compromising material strength. The high temperatures necessary to mould polymers led to significant drug loss [205]. Sullivan *et al.* proposed a photo-polymerization method to use UV light to form microneedles without compromising the activity of β-galactosidase [207].

Two companies, TheraJect and BioSerenTach (figure 6J) are currently developing dissolvable microneedle systems for vaccine delivery. The TheraJect VaxMat®, made of a sugar matrix containing vaccine components, 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 a few minutes [208]. Given water-proof packaging, fast-dissolving microneedle arrays provide a one-step solution for TCI.

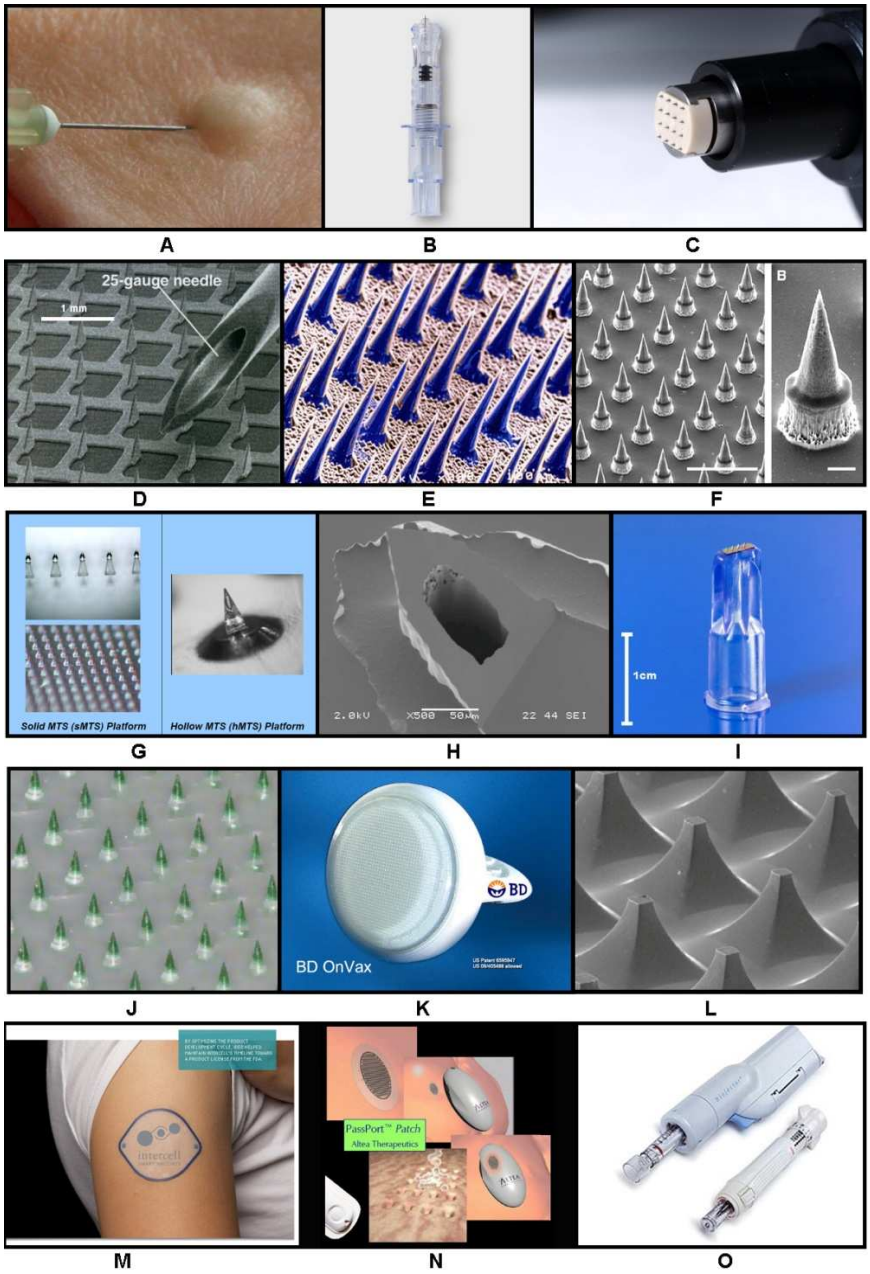


Figure 6. Examples of approaches and devices used for ID immunisation (A, B) and TCI (C-O). (A) classical ID immunisation; (B) Soluvia™ (BD) [192]; (C) Applicator with a solid microneedle array as used in [171]; (D) solid microneedles of the Macroflux® [176]; (E) array of silicon microneedles [169]; (F) Coated microneedles [183]; (G) coated and hollow microneedle arrays (3M); (H) silicon hollow microneedle [127]; (I) hollow microneedle array, MicronJet® (NanoPass) [156]; (J) dissolvable microneedle array from BioSerenTach [193]; (K &L) blunt-tipped microneedle array, OnVax® (BD) and its electron microscopy image [194]; (M) smart vaccine patch from Intercell [195]; (N) PassPort™ patch (Altea) [196]; (O) powder jet systems, adapted from [197].

Other microneedle arrays

BD's OnVax® device employs blunt-tipped microneedles being 50–200 µm in length over a 1 cm^2 area (figure 6K and L). These "microenhancer arrays" were used to gently scrape the skin containing a vaccine solution in order to expose the epidermis to the vaccine without pain sensation [194]. Using a hepatitis B DNA vaccine (100 µg dose), stronger and less variable immune responses were achieved compared to IM and ID injection with the same dose. Moreover, 100% of seroconversion was achieved after only two immunisations, whereas only 40-50% conversion was obtained by the conventional techniques. This enables "wipe and go" vaccination with easy self-administration [194]. However, although DNA vaccines can be produced in larger quantity with lower costs compared to subunit vaccines, the amount of vaccine delivered using this device is very low.

The EasyVax™ device has been designed to insert coated microneedle arrays into the skin followed by electrical pulses to deliver DNA into the cells (figure 6M). Following this procedure, TCI with a smallpox DNA vaccine induced neutralizing antibody titres greater than those elicited by the traditional live virus vaccine administered by scarification [209]. Even though the animal studies with the EasyVax™ are promising, the main drawback of this approach is the complexity of the device.

The use of an applicator

Verbaan *et al.* showed that 300 µm long microneedles were not able to pierce the skin when applied manually. It was found that the elasticity of the skin results in folding of the skin surrounding the microneedles [210]. Consequently, an electric applicator, providing an injection speed of 3 m/s, enabled the 300 µm long microneedle arrays, and even the 245 µm long ones to pierce the skin effectively and reproducibly (figure 6C) [171]. Crichton *et al.* showed that, by varying the application speed of coated microneedles, the amount of microneedles piercing the skin and the delivered dose can be increased [183]. More importantly, they showed that the antigen can be targeted either to the epidermis or to both the epidermis and the dermis, so one can decide whether to deliver the majority of the vaccine only to the LCs or also to the dDCs. These studies highlighted the necessity of an applicator. It is conceivable that a higher velocity is needed to counteract the elasticity and ensure efficient penetration of the skin. A mechanical applicator device is superior to manual application as it can provide an adjustable yet consistent projection speed, with minimal inter-individual variability. Applicators available on the market or under development are either integrated with the microneedle patch or supplied as a separate device, for single or repeated use, respectively [154]. It is possible to pierce manually using longer microneedles, but with a less precise penetration depth.

Some trends can be noticed from studies performed during the last ten years in this field:

i) instead of piercing on dermatomed skin *in vitro*, since recently very relevant experimental evaluations are being performed also *in vivo;*
ii) an impact applicator or insertion device is often used. It provides defined projection speed (faster than applied manually) of the microneedle arrays, thereby enhancing the uniformity of skin piercing and allowing shorter needle lengths;
iii) coated microneedle arrays (as well as dissolvable ones) may provide TCI with simple patch design, resulting in competitive products on the market;
iv) hollow microneedle arrays have gained more attention for their potential of precise dose control, while the device design needs to be improved with respect to leakage-free injection and simplicity;
v) antigen doses used in TCI fall in broad range depending on the animal model and the delivery methods. Studies on dose-dependency and dose refinement should be included in future TCI studies.

Other approaches

Besides microneedles, a large number of approaches have emerged to overcome the skin barrier. These methods have been reviewed extensively elsewhere [167, 211, 212] and we will shortly discuss the most promising techniques for TCI (Table 4B).

One way to overcome the stratum corneum barrier is to remove it by tape-stripping, abrasion or thermo-ablation. Glenn *et al.* were a pioneer in this field, showing that mild abrasion results in the removal of approximately 29% of the stratum corneum, which greatly enhances the passive diffusion of an antigen. Stratum corneum disruption prior to applying a vaccine patch (containing 50 µg LT) resulted in IgG titres comparable to those obtained after active toxin infection and those induced by oral cholera vaccine [213, 214]. Later on they developed the Skin Preparation System (SPS) which was successful in phase I and II studies against traveller's diarrhoea [149, 215, 216] and has currently entered phase III development (figure 6M). This would be the first vaccine delivered with a patch on the market. The mechanism of action of these patches partly depends on occlusion of the skin they cover, which increases the hydration of the skin. Increased hydration progressively increases its permeability, due to swelling of the corneocytes, pooling of fluid in the intercellular spaces and dramatic microscopic changes in its structure at very high hydration levels [217]. The PassPort^{TM} patch system (figure 6N), developed by Altea, creates 80 micropores within a 1 cm^2 area using thermo-ablation [218]. An applicator is employed to release a single pulse of energy. TCI using this system by application of a prime and two booster vaccinations with 3 µg doses of recombinant H5 influenza hemagglutinin adjuvanted with 25 µg CpG 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 [218].

Besides heat, ultrasound and electrical pulses have also been used to disturb the stratum corneum. These techniques have not yet been used extensively, due to complicated devices which still need to be optimized. However, preliminary studies show that both methods are able to induce an immune response, although with relatively high antigen and adjuvant doses [219, 220].

Finally, vaccines can be delivered by powder or liquid jet injections. A lot of studies have been performed using epidermal powder immunisation, showing protective immune responses against influenza, hepatitis B and DT with doses ranging from 0.2 – 5 µg [140, 221-223]. This device is now acquired by Pfizer (PMED™, figure 6O) to target dry powder DNA vaccines to mainly the epidermis of the skin [224-229]. The high impact with which very small sugar or gold coated particles enter the skin will disrupt cells, thereby inducing LC activation and migration from the skin in a similar fashion as after microneedle application [222]. This disruption causes mild side effects, such as application site burning, which usually resolves within hours [225, 229]. Liquid jet injections, very popular until the 1985 hepatitis outbreak [230], have now regained interest with safer design, e.g. disposable cartridges prefilled with vaccines [231].

Table 4. New technologies targeting vaccine delivery into the skin.

A: Microneedle-related approaches

Technology	Vaccine (development phase)	Company or Ref
Hollow needles (ID)		
• Soluvia™	Trivalent inactivated seasonal influenza vaccine (phase III)	BD/Sanofi-Pasteur, [159]
• Nanoject	not available	Debiotech
• other systems	recombinant protective anthrax vaccine (pre-clinical)	BD, [232]
Hollow microneedles (TCI)		
• MicronJet®	Trivalent subunit (HA) seasonal influenza vaccine (phase I)	NanoPass, [156]
Solid microneedles		
• MTS*	OVA, M2E-flagellin influenza subunit vaccine (pre-clinical)	3M & VaxInnate [175]
• OnVax®	Hepatitis B DNA vaccine (pre-clinical)	BD, [194]
• other systems	DT, influenza subunit vaccine (pre-clinical)	[126, 127, 130, 172]

Coated microneedles		
• Macroflux®	Influenza vaccine (phase I)	Zosano (Alza)
	OVA (pre-clinical)	[163, 176]
• MTS	not available	3M
• other systems	OVA, hepatitis C DNA vaccine, inactivated influenza virus (pre-clinical)	[178-183]
Dissolvable microneedles		
• VaxMat®	not available	TheraJect
• other systems	Inactivated influenza vaccine	BioSerenTach, [193, 206]
Microneedles with electroporation		
• EasyVax®	Smallpox DNA vaccine (pre-clinical)	[209]

B: Other physical and chemical approaches

Technology	**Vaccine/(development phase)**	**Company or Ref**
Skin abrasion		
• SPS**	Trivalent inactivated seasonal influenza (phase II)	Iomai/Intercell
	LT for travelers' diarrhea (phase III)	[233, 234]
	Virosomal influenza subunit vaccine (clinical phase II)	[235]
	Recombinant protective anthrax antigen (pre-clinical)	[236]
• CSSS***	Inactivated influenza/tetanus vaccine or subunit influenza vaccine(phase I)	[237, 238]
	DT (pre-clinical)	[121]
	Melanoma or HIV epitopes (phase I)	[239]
	Vaccinia Ankara (pre-clinical)	[240]
Low frequency (20 kHz) ultrasound	TT (pre-clinical)	[219]
Electroporation		
• Elgen® / CELLECTRA®	HIV & influenza DNA vaccines	Inovio, [241, 242]
• other systems	OVA peptide (pre-clinical), pGL3 luciferase DNA	[220, 243]

Thermo-ablation		
• PassPort™ system	(recombinant) influenza protein (pre-clinical)	Altea, [218]
Jet immunisation		
• PMED™ (powder)	HIV DNA vaccine (pre-clinical)	[226]
	HSV**** type 2 DNA vaccine (phase I)	[228]
	DNA melanoma gp100 (phase I)	[227]
	Influenza DNA vaccine (phase I)	[225]
	Hepatitis B DNA vaccine (phase II)	[224]
	Influenza DNA vaccine (phase I)	[229]
	Malaria DNA vaccine (phase I)	[244, 245]
• Biojector® 2000 (liquid)	HIV DNA vaccine (phase I)	Bioject, [246]
	Inactivated hepatitis A vaccine(phase I)	[109]
	Rotavirus, Dengue DNA (pre-clinical)	[247, 248]
	Inactivated polio vaccine (phase I)	
• PharmaJet® (liquid)	Measles-mumps-rubella	PharmaJet Inc.
	Yellow fever	
• Mini-ject	not available	Valeritas

* MTS: microstructured transdermal system
** SPS: Skin preparation system
*** CSSS: cyanoacrylate skin surface stripping
**** HSV: herpes simplex virus

The stages of development of the approaches mentioned are summarized in Table 4. The long list of strategies/devices, developed to overcome the skin barrier and enable painless TCI, reflects a very competitive and fast developing field. Combining techniques might be necessary to target the preferred APCs. 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 an antigen to a single skin DC subset.

Design of novel formulations

Formulation of antigens in particulate carriers is a popular strategy to improve vaccine delivery, also via the transcutaneous route [249, 250]. The usage of nanoparticles as antigen carriers has several advantages. They can retain the antigen at the delivery site for a prolonged period [4] and improve the uptake of antigens by APCs, because of their similar size and structure to microorganisms, the natural pathogens which are actively sampled by the APCs [251]. Another advantage is the possibility to encapsulate both antigen and adjuvant in the same particle, which is suggested to enhance the immunogenicity [252]. However, the usage of nanoparticles for TCI so far is limited. The focus has mainly been on lipid vesicles, *i.e.* closed spherical structures consisting of bilayers of hydrated amphiphilic lipids or other amphiphilic compounds. 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 [253, 254]. In particular, elastic vesicles, which have a flexible bilayer, have been used as they are supposed to penetrate the stratum corneum more easily as compared to conventional liposomes.

Transfersomes®

Transfersomes® are ultra-deformable liposomes, generated by incorporation of a surfactant in the lipid bilayer [255, 256]. Transfersomes® are applied non-occlusively as it has been suggested that the hydration gradient in the stratum corneum will drive the intact vesicles into the viable epidermis [257]. However, this claim has not yet been substantiated [258]. Nevertheless, several groups have independently reported that Transfersomes® substantially increase the transport of small molecules across the stratum corneum [255, 259-261].

The use of Transfersomes® to formulate antigens in TCI has also been reported in a few studies. When using antigens such as human serum albumin, gap junction protein and TT, potent humoral immune responses were induced in murine models with antibody levels comparable to those obtained through SC injection [262-264]. Transfersomes® prepared with soybean phosphatidylcholine (PC), Span 80 and ethanol, were loaded with hepatitis B surface antigen (HBsAg). Comparable IgG titres and much higher secretory IgA titters against HBsAg were induced when elastic liposomes loaded with 10 µg HBsAg were applied onto intact mouse skin as compared to those obtained by IM injection of the same dose of alum-adsorbed HBsAg [265]. However, in these studies no washing step was included after topical antigen application on the back of the animals to remove the remaining formulations. This raises the question if the immune responses were purely induced by TCI or if oral delivery also contributed, *e.g.* through grooming of the rodents. In contrast in our group elastic cationic liposomes made of PC, Span 80 and DOTAP (1,2-dioleoyl-3-

trimethylammonium-propane chloride salt) did not improve the immune response when loaded with DT and applied topically for 1 hour on intact mouse skin while the mice were kept under anaesthesia [172].

Other elastic vesicles

Van den Bergh et al. introduced a series of surfactant-based elastic vesicles, consisting of a bilayer-forming surfactant (sucrose-laurate ester), a surfactant (octaoxyethylene-laurate ester) and a charge inducer (sodium bistridecyl sulfo succinate) [266, 267]. Enhanced transdermal diffusion through intact skin of low-molecular weight drugs incorporated in elastic vesicles has been observed and vesicular structures were visualized in deep layers of the stratum corneum [268-270]. However, in the TCI studies using elastic vesicle-incorporated DT on intact mouse skin, no enhanced immune response compared to a DT solution was induced [172]. Other generations of elastic vesicles 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. TCI of HBsAg-loaded ethosomes (composed of soybean PC and ethanol) has been reported to induce immune response comparable to IM injection of HBsAg-alum [271]. BSA-loaded niosomes, composed of Span 60, Span 85, cholesterol and stearylamine, were coated with a modified polysaccharide O-palmitoyl mannan for targeted delivery to the LCs. This niosomal formulation elicited significantly higher serum IgG titres as compared with alum-adsorbed BSA and plain uncoated niosomes in TCI, but still lower than those obtained after IM injection of an equivalent dose of BSA-alum [272].

Non-elastic nanoparticles

Besides elastic vesicles a modest number of groups have investigated the use of polymeric nanoparticles for TCI, so far with limited success. Not surprisingly, Mattheolabakis et al. found no advantage of antigen encapsulation in negatively charged polylactic acid (PLA) nanoparticles when applied on intact skin [273]. Much smaller virus-like particles (40 nm), when adjuvanted with CpG were able to induce humoral and cellular immune responses [274]. To overcome the skin barrier we applied DT-loaded N-trimethyl chitosan (TMC) nanoparticles on microneedle pre-treated skin [130]. Applying these nanoparticles for one hour did not enhance the immune response compared to a DT solution. However, using a longer application time, the nanoparticles were more efficient in potentiating the immune response than a DT solution showing that TMC nanoparticle diffusion might be an important limiting factor for the potency in TCI (unpublished results). Conjugating the antigen to the polymer, thereby creating a smaller unit, could further increase the potential of TMC [275-277]. In related in vitro studies it was shown that TMC itself acts as an adjuvant and stimulates DC maturation [278, 279].

Other formulation issues

The delivery of nanoparticles in TCI needs to be further optimized and studies on the effect of size, charge and intrinsic adjuvant properties of particulate carrier systems are needed. Another important issue in the development of formulations for TCI is antigen stability. As previously mentioned, coating of vaccines onto microneedles and the formulation into biodegradable microneedles can affect the stability and effectiveness of the vaccine. Similarly, encapsulation of antigens in nanoparticles may compromise their stability and antigenicity. Moreover, when particulate antigen carriers are used, the colloidal stability of the formulation should be addressed.

Limitations of animal models

The physiological differences between lab animals and humans should be taken into consideration when transferring techniques of skin barrier disruption between species. For example, skin of humans and mice is similarly densely populated with immune active cells, especially LCs [280, 281]. However, human skin is much thicker and less hairy than mouse skin. The epidermis of human skin is approximately 150 µm thick, compared to only 10 µm in mice [282, 283]. Correspondingly, human LCs are located deeper in the skin [280]. This fact is often not considered in the development of (micro)needle devices. Needle-lengths vary considerably, up to 1 mm in length. The total skin thickness in mice is about 500 µm [283], so if longer needles are used in mice studies they may reach the subcutaneous tissue in addition to the dermis. Another difference between human skin and mouse skin is that the latter is more hairy and consequently has more hair follicles. It has been shown that hair follicles can be used for drug delivery [284] and nanoparticles were shown to accumulate in the hair follicles and be taken up by surrounding APCs [240, 273] . TCI via the hair follicles is also possible in human skin, as recently a clinical phase I study showed induction of $CD8^+$ T cells after immunisation with an inactivated influenza vaccine [237].
Finally, an important limitation of animal models is the restricted application time of vaccines. Usually animals need to be anesthetized to prevent them from grooming and oral vaccine delivery. Several of the particulate formulations mentioned above were only applied for 1 hour [130, 172]. In humans patches can easily be left on the skin for up to 24 hours. These factors need to be taken into account when designing vaccination studies and interpreting the data.

Concluding remarks

The skin is an important immunological site and, although it poses a complex barrier, has the potential to be an ideal non-invasive vaccination site. TCI provides effective, easy-to-use and painless vaccination with fewer side effects and safer handling than the

conventional injections. The main challenges are to ensure accurate delivery of antigens into the epidermal and/or dermal skin tissue and to formulate antigens with adjuvants and/or carrier systems for selective activation of the proper PRRs existing in the skin DC subsets. Many different approaches have been developed of which several ways may lead to successful TCI (table 4). The most promising systems combine barrier disruption (most probably with microneedles) with the addition of an adjuvant to the vaccine formulation. For particulate formulations to be successful, this barrier disruption is of crucial importance. However the most efficient way still needs to be found, which will require joint efforts from immunologists, vaccinologists, pharmaceutical scientists and (fine) mechanical engineers. Only then TCI can be further improved and essentially revolutionize the current vaccination practice. The ideal strategy is to combine skin barrier disruption approaches with use of adjuvants. 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.

Acknowledgements

This research was performed under the framework of TI Pharma project number D5-106-1; Vaccine delivery: alternatives for conventional multiple injection vaccines. The authors thank Aat Mulder for providing the electron microscopy picture of the skin.

References

1. Hilleman MR, *Vaccines in historic evolution and perspective: a narrative of vaccine discoveries.* Vaccine, 2000. **18**(15): p. 1436-47.
2. Ada G, *Overview of vaccines.* Methods Mol Med, 2003. **87**: p. 1-17.
3. Schijns VE, *Immunological concepts of vaccine adjuvant activity.* Curr Opin Immunol, 2000. **12**(4): p. 456-63.
4. Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, and Narasimhan B, *Vaccine adjuvants: current challenges and future approaches.* J Pharm Sci, 2009. **98**(4): p. 1278-316.
5. Jacobson RM, Swan A, Adegbenro A, Ludington SL, Wollan PC, Poland GA, and Grp VR, *Making vaccines more acceptable - methods to prevent and minimize pain and other common adverse events associated with vaccines.* Vaccine, 2001. **19**(17-19): p. 2418-2427.
6. Slutter B, Hagenaars N, and Jiskoot W, *Rational design of nasal vaccines.* J Drug Target, 2008. **16**(1): p. 1-17.
7. Mikszta JA and Laurent PE, *Cutaneous delivery of prophylactic and therapeutic vaccines: historical perspective and future outlook.* Expert Rev Vaccines, 2008. **7**(9): p. 1329-39.
8. Simerska P, Moyle PM, Olive C, and Toth I, *Oral vaccine delivery--new strategies and technologies.* Curr Drug Deliv, 2009. **6**(4): p. 347-58.
9. Lu D and Hickey AJ, *Pulmonary vaccine delivery.* Expert Rev Vaccines, 2007. **6**(2): p. 213-26.
10. Henderson DA, L.L. B, and Grabenstein JD, *Smallpox and vaccinia*, in *Vaccines*, Plotkin S.A., Orenstein W.A., and Offit P.A., Editors. 2008, Elsevier: Amsterdam. p. 773-803.
11. Kupper TS and Fuhlbrigge RC, *Immune surveillance in the skin: mechanisms and clinical consequences.* Nat Rev Immunol, 2004. **4**(3): p. 211-22.
12. Ponvert C and Scheinmann P, *Vaccine allergy and pseudo-allergy.* Eur J Dermatol, 2003. **13**(1): p. 10-5.
13. Bos JD and Meinardi MM, *The 500 Dalton rule for the skin penetration of chemical compounds and drugs.* Exp Dermatol, 2000. **9**(3): p. 165-9.
14. Schaefer H and Redelmeier TE, *Principles of percutaneous absorption* in *Skin barrier*. 1996, Karger: Basel.
15. Barry BW, *Percutaneous absorption*, in *Dermatological formulations* 1983, Marcel Dekker Inc.: New York.
16. Watt FM, *Terminal differentiation of epidermal keratinocytes.* Curr Opin Cell Biol, 1989. **1**(6): p. 1107-15.
17. Yamamura M, Uyemura K, Deans RJ, Weinberg K, Rea TH, Bloom BR, and Modlin RL, *Defining protective responses to pathogens: cytokine profiles in leprosy lesions.* Science, 1991. **254**(5029): p. 277-9.
18. Matzinger P, *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
19. Asahina A and Tamaki K, *Role of Langerhans cells in cutaneous protective immunity: is the reappraisal necessary?* J Dermatol Sci, 2006. **44**(1): p. 1-9.
20. Partidos CD and Muller S, *Decision-making at the surface of the intact or barrier disrupted skin: potential applications for vaccination or therapy.* Cell Mol Life Sci, 2005. **62**(13): p. 1418-24.
21. Kissenpfennig A and Malissen B, *Langerhans cells--revisiting the paradigm using genetically engineered mice.* Trends Immunol, 2006. **27**(3): p. 132-9.
22. Sugita K, Kabashima K, Koga C, and Tokura Y, *Eosinophilic pustular folliculitis successfully treated with sequential therapy of interferon-gamma and ciclosporin.* Clin Exp Dermatol, 2006. **31**(5): p. 709-10.
23. Schmuth M, Neyer S, Rainer C, Grassegger A, Fritsch P, Romani N, and Heufler C, *Expression of the C-C chemokine MIP-3 alpha/CCL20 in human epidermis with impaired permeability barrier function.* Exp Dermatol, 2002. **11**(2): p. 135-42.
24. Dieu-Nosjean MC, Massacrier C, Homey B, Vanbervliet B, Pin JJ, Vicari A, Lebecque S, Dezutter-Dambuyant C, Schmitt D, Zlotnik A, and Caux C, *Macrophage inflammatory protein 3 alpha is*

expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. J Exp Med, 2000. **192**(5): p. 705-717.
25. Nickoloff BJ and Turka LA, *Immunological functions of nonprofessional antigen-presenting cells - New insights from studies of T-Cell interactions with keratinocytes.* Immunology Today, 1994. **15**(10): p. 464-469.
26. Ludwig IS, Geijtenbeek TB, and van Kooyk Y, *Two way communication between neutrophils and dendritic cells.* Curr Opin Pharmacol, 2006. **6**(4): p. 408-13.
27. Malaviya R, Ikeda T, Ross E, and Abraham SN, *Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha.* Nature, 1996. **381**(6577): p. 77-80.
28. Luci C, Reynders A, Ivanov, II, Cognet C, Chiche L, Chasson L, Hardwigsen J, Anguiano E, Banchereau J, Chaussabel D, Dalod M, Littman DR, Vivier E, and Tomasello E, *Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin.* Nat Immunol, 2009. **10**(1): p. 75-82.
29. Banchereau J and Steinman RM, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.
30. Akira S, Uematsu S, and Takeuchi O, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
31. Medzhitov R, Preston-Hurlburt P, and Janeway CA, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.
32. van Duin D, Medzhitov R, and Shaw AC, *Triggering TLR signaling in vaccination.* Trends Immunol, 2006. **27**(1): p. 49-55.
33. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, and Wagner H, *Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments.* Eur J Immunol, 2002. **32**(7): p. 1958-68.
34. van der Aar AM, Sylva-Steenland RM, Bos JD, Kapsenberg ML, de Jong EC, and Teunissen MB, *Loss of TLR2, TLR4, and TLR5 on Langerhans cells abolishes bacterial recognition.* J Immunol, 2007. **178**(4): p. 1986-90.
35. Flacher V, Bouschbacher M, Verronese E, Massacrier C, Sisirak V, Berthier-Vergnes O, de Saint-Vis B, Caux C, Dezutter-Dambuyant C, Lebecque S, and Valladeau J, *Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria.* J Immunol, 2006. **177**(11): p. 7959-67.
36. Klechevsky E, Liu M, Morita R, Banchereau R, Thompson-Snipes L, Palucka AK, Ueno H, and Banchereau J, *Understanding human myeloid dendritic cell subsets for the rational design of novel vaccines.* Hum Immunol, 2009. **70**(5): p. 281-8.
37. Mempel M, Voelcker V, Kollisch G, Plank C, Rad R, Gerhard M, Schnopp C, Fraunberger P, Walli AK, Ring J, Abeck D, and Ollert M, *Toll-like receptor expression in human keratinocytes: nuclear factor kappaB controlled gene activation by Staphylococcus aureus is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent.* J Invest Dermatol, 2003. **121**(6): p. 1389-96.
38. Kollisch G, Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J, Bauer S, Jakob T, Mempel M, and Ollert M, *Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes.* Immunology, 2005. **114**(4): p. 531-41.
39. Miller LS, Sorensen OE, Liu PT, Jalian HR, Eshtiaghpour D, Behmanesh BE, Chung W, Starner TD, Kim J, Sieling PA, Ganz T, and Modlin RL, *TGF-alpha regulates TLR expression and function on epidermal keratinocytes.* J Immunol, 2005. **174**(10): p. 6137-43.
40. Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitemaker JH, Kapsenberg ML, and de Jong EC, *Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9.* J Invest Dermatol, 2007. **127**(2): p. 331-41.
41. Yu N, Zhang S, Zuo F, Kang K, Guan M, and Xiang L, *Cultured human melanocytes express functional toll-like receptors 2-4, 7 and 9.* J Dermatol Sci, 2009. **56**(2): p. 113-20.
42. Miller LS and Modlin RL, *Toll-like receptors in the skin.* Semin Immunopathol, 2007. **29**(1): p. 15-26.

43. Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, and Banchereau J, *Dendritic cell subsets in health and disease.* Immunol Rev, 2007. **219**: p. 118-42.
44. Welte T, Reagan K, Fang H, Machain-Williams C, Zheng X, Mendell N, Chang GJ, Wu P, Blair CD, and Wang T, *Toll-like receptor 7-induced immune response to cutaneous West Nile virus infection.* J Gen Virol, 2009. **90**(Pt 11): p. 2660-8.
45. Mitsui H, Watanabe T, Saeki H, Mori K, Fujita H, Tada Y, Asahina A, Nakamura K, and Tamaki K, *Differential expression and function of Toll-like receptors in Langerhans cells: comparison with splenic dendritic cells.* J Invest Dermatol, 2004. **122**(1): p. 95-102.
46. Waibler Z, Kalinke U, Will J, Juan MH, Pfeilschifter JM, and Radeke HH, *TLR-ligand stimulated interleukin-23 subunit expression and assembly is regulated differentially in murine plasmacytoid and myeloid dendritic cells.* Mol Immunol, 2007. **44**(7): p. 1483-9.
47. Kelsall BL, Biron CA, Sharma O, and Kaye PM, *Dendritic cells at the host-pathogen interface.* Nat Immunol, 2002. **3**(8): p. 699-702.
48. Boonstra A, Rajsbaum R, Holman M, Marques R, Asselin-Paturel C, Pereira JP, Bates EE, Akira S, Vieira P, Liu YJ, Trinchieri G, and O'Garra A, *Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals.* J Immunol, 2006. **177**(11): p. 7551-8.
49. von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, Chrabieh M, Mustapha IB, Ghandil P, Camcioglu Y, Vasconcelos J, Sirvent N, Guedes M, Vitor AB, Herrero-Mata MJ, Arostegui JI, Rodrigo C, Alsina L, Ruiz-Ortiz E, Juan M, Fortuny C, Yague J, Anton J, Pascal M, Chang HH, Janniere L, Rose Y, Garty BZ, Chapel H, Issekutz A, Marodi L, Rodriguez-Gallego C, Banchereau J, Abel L, Li X, Chaussabel D, Puel A, and Casanova JL, *Pyogenic bacterial infections in humans with MyD88 deficiency.* Science, 2008. **321**(5889): p. 691-6.
50. Ting JP and Davis BK, *Caterpiller: a novel gene family important in immunity, cell death, and diseases.* Annu Rev Immunol, 2005. **23**: p. 387-414.
51. Martinon F and Tschopp J, *NLRs join TLRs as innate sensors of pathogens.* Trends Immunol, 2005. **26**(8): p. 447-54.
52. McDonald C, Inohara N, and Nunez G, *Peptidoglycan signaling in innate immunity and inflammatory disease.* J Biol Chem, 2005. **280**(21): p. 20177-80.
53. Valiante N, De Gregorio E, and Rappuoli R, *Toll-free immunity?* Nat Med, 2008. **14**(12): p. 1318-1319.
54. Figdor CG, van Kooyk Y, and Adema GJ, *C-type lectin receptors on dendritic cells and Langerhans cells.* Nat Rev Immunol, 2002. **2**(2): p. 77-84.
55. Geijtenbeek TB, van Vliet SJ, Engering A, t Hart BA, and van Kooyk Y, *Self- and nonself-recognition by C-type lectins on dendritic cells.* Annu Rev Immunol, 2004. **22**: p. 33-54.
56. Kapsenberg ML, *Dendritic-cell control of pathogen-driven T-cell polarization.* Nat Rev Immunol, 2003. **3**(12): p. 984-93.
57. Janeway CA, Jr. and Medzhitov R, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
58. Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ, and Frucht DM, *Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights.* J Clin Immunol, 2003. **23**(3): p. 147-61.
59. Mosmann TR and Coffman RL, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. **7**: p. 145-73.
60. Langenkamp A, Messi M, Lanzavecchia A, and Sallusto F, *Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells.* Nat Immunol, 2000. **1**(4): p. 311-6.
61. Steinman L, *A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage.* Nat Med, 2007. **13**(2): p. 139-45.
62. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, and Mackay CR, *T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells.* J Immunol, 2004. **173**(1): p. 68-78.

63. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, and Stockinger B, *Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset.* Nat Immunol, 2008. **9**(12): p. 1341-6.
64. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, Mitsdoerffer M, Strom TB, Elyaman W, Ho IC, Khoury S, Oukka M, and Kuchroo VK, *IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells.* Nat Immunol, 2008. **9**(12): p. 1347-55.
65. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, and Sallusto F, *Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells.* Nat Immunol, 2009. **10**(8): p. 857-63.
66. Trifari S, Kaplan CD, Tran EH, Crellin NK, and Spits H, *Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells.* Nat Immunol, 2009. **10**(8): p. 864-71.
67. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, and Melief CJ, *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions.* Nature, 1998. **393**(6684): p. 480-3.
68. Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, and Zinkernagel RM, *The influence of antigen organization on B cell responsiveness.* Science, 1993. **262**(5138): p. 1448-51.
69. Kelsoe G, *Studies of the humoral immune response.* Immunol Res, 2000. **22**(2-3): p. 199-210.
70. Sallusto F and Lanzavecchia A, *Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity.* Eur J Immunol, 2009. **39**(8): p. 2076-82.
71. Clark RA, Chong B, Mirchandani N, Brinster NK, Yamanaka K, Dowgiert RK, and Kupper TS, *The vast majority of CLA+ T cells are resident in normal skin.* J Immunol, 2006. **176**(7): p. 4431-9.
72. Puissant-Lubrano B, Bossi P, Gay F, Crance JM, Bonduelle O, Garin D, Bricaire F, Autran B, and Combadiere B, *Control of vaccinia virus skin lesions by long-term-maintained IFN-gamma+TNF-alpha+ effector/memory CD4+ lymphocytes in humans.* J Clin Invest. **120**(5): p. 1636-44.
73. Liu L, Zhong Q, Tian T, Dubin K, Athale SK, and Kupper TS, *Epidermal injury and infection during poxvirus immunization is crucial for the generation of highly protective T cell-mediated immunity.* Nat Med, 2010. **16**(2): p. 224-7.
74. Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, and Carbone FR, *Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus.* Nat Immunol, 2009. **10**(5): p. 524-30.
75. Clark RA, *Skin-resident T cells: the ups and downs of on site immunity.* J Invest Dermatol, 2010. **130**(2): p. 362-70.
76. Piqueras B, Connolly J, Freitas H, Palucka AK, and Banchereau J, *Upon viral exposure, myeloid and plasmacytoid dendritic cells produce 3 waves of distinct chemokines to recruit immune effectors.* Blood, 2006. **107**(7): p. 2613-8.
77. Renn CN, Sanchez DJ, Ochoa MT, Legaspi AJ, Oh CK, Liu PT, Krutzik SR, Sieling PA, Cheng G, and Modlin RL, *TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response.* J Immunol, 2006. **177**(1): p. 298-305.
78. Medzhitov R and Janeway C, Jr., *Innate immunity.* N Engl J Med, 2000. **343**(5): p. 338-44.
79. Medzhitov R, *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.
80. Medzhitov R and Janeway CA, Jr., *Innate immunity: impact on the adaptive immune response.* Curr Opin Immunol, 1997. **9**(1): p. 4-9.
81. Nestle FO, Di Meglio P, Qin JZ, and Nickoloff BJ, *Skin immune sentinels in health and disease.* Nat Rev Immunol, 2009. **9**(10): p. 679-91.
82. Heath WR and Carbone FR, *Dendritic cell subsets in primary and secondary T cell responses at body surfaces.* Nat Immunol, 2009. **10**(12): p. 1237-44.
83. Teunissen MB, Kapsenberg ML, and Dos JD, *Langerhans cells and related skin dendritic cells. In: Skin immune system*. 2nd ed. 1997: CRC Press LLC.
84. Valladeau J, Ravel O, Dezutter-Dambuyant C, Moore K, Kleijmeer M, Liu Y, Duvert-Frances V, Vincent C, Schmitt D, Davoust J, Caux C, Lebecque S, and Saeland S, *Langerin, a novel C-type lectin specific to*

Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. Immunity, 2000. **12**(1): p. 71-81.

85. Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, Bloom BR, Brenner MB, Kronenberg M, Brennan PJ, and Modlin RL, *Cd1-restricted T-cell recognition of microbial lipoglycan antigens.* Science, 1995. **269**(5221): p. 227-230.
86. Valladeau J and Saeland S, *Cutaneous dendritic cells.* Semin Immunol, 2005. **17**(4): p. 273-83.
87. Zaba LC, Fuentes-Duculan J, Steinman RM, Krueger JG, and Lowes MA, *Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163+FXIIIA+ macrophages.* J Clin Invest, 2007. **117**(9): p. 2517-25.
88. Nestle FO, Zheng XG, Thompson CB, Turka LA, and Nickoloff BJ, *Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets.* J Immunol, 1993. **151**(11): p. 6535-6545.
89. Poulin LF, Henri S, de Bovis B, Devilard E, Kissenpfennig A, and Malissen B, *The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells.* J Exp Med, 2007. **204**(13): p. 3119-31.
90. Ginhoux F, Collin MP, Bogunovic M, Abel M, Leboeuf M, Helft J, Ochando J, Kissenpfennig A, Malissen B, Grisotto M, Snoeck H, Randolph G, and Merad M, *Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state.* J Exp Med, 2007. **204**(13): p. 3133-46.
91. Bursch LS, Wang L, Igyarto B, Kissenpfennig A, Malissen B, Kaplan DH, and Hogquist KA, *Identification of a novel population of Langerin(+) dendritic cells.* J Exp Med, 2007. **204**(13): p. 3147-3156.
92. Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, Allan RS, Wojtasiak M, Shortman K, Carbone FR, Brooks AG, and Heath WR, *Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells.* Nat Immunol, 2009. **10**(5): p. 488-95.
93. Brewig N, Kissenpfennig A, Malissen B, Veit A, Bickert T, Fleischer B, Mostbock S, and Ritter U, *Priming of CD8(+) and CD4(+) T cells in experimental leishmaniasis is initiated by different dendritic cell subtypes.* J Immunol, 2009. **182**(2): p. 774-783.
94. Nagao K, Ginhoux F, Leitner WW, Motegi S, Bennett CL, Clausen BE, Merad M, and Udey MC, *Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3312-7.
95. Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, Briere F, Chaussabel D, Zurawski G, Palucka AK, Reiter Y, Banchereau J, and Ueno H, *Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells.* Immunity, 2008. **29**(3): p. 497-510.
96. Hunger RE, Sieling PA, Ochoa MT, Sugaya M, Burdick AE, Rea TH, Brennan PJ, Belisle JT, Blauvelt A, Porcelli SA, and Modlin RL, *Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells.* J Clin Invest, 2004. **113**(5): p. 701-8.
97. Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, and Figdor CG, *Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses.* Cell, 2000. **100**(5): p. 575-85.
98. Angel CE, Lala A, Chen CJ, Edgar SG, Ostrovsky LL, and Dunbar PR, *CD14+ antigen-presenting cells in human dermis are less mature than their CD1a+ counterparts.* Int Immunol, 2007. **19**(11): p. 1271-9.
99. Caux C, Massacrier C, Vanbervliet B, Dubois B, Durand I, Cella M, Lanzavecchia A, and Banchereau J, *CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis.* Blood, 1997. **90**(4): p. 1458-70.
100. Dubois B, Massacrier C, Vanbervliet B, Fayette J, Briere F, Banchereau J, and Caux C, *Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes.* J Immunol, 1998. **161**(5): p. 2223-31.
101. Itano AA, McSorley SJ, Reinhardt RL, Ehst BD, Ingulli E, Rudensky AY, and Jenkins MK, *Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity.* Immunity, 2003. **19**(1): p. 47-57.

102. Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N, Tripp CH, Douillard P, Leserman L, Kaiserlian D, Saeland S, Davoust J, and Malissen B, *Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells.* Immunity, 2005. **22**(5): p. 643-54.
103. Ratzinger G, Baggers J, de Cos MA, Yuan J, Dao T, Reagan JL, Munz C, Heller G, and Young JW, *Mature human Langerhans cells derived from CD34+ hematopoietic progenitors stimulate greater cytolytic T lymphocyte activity in the absence of bioactive IL-12p70, by either single peptide presentation or cross-priming, than do dermal-interstitial or monocyte-derived dendritic cells.* J Immunol, 2004. **173**(4): p. 2780-91.
104. Banchereau J, Klechevsky E, Schmitt N, Morita R, Palucka K, and Ueno H, *Harnessing human dendritic cell subsets to design novel vaccines.* Ann Ny Acad Sci, 2009. **1174**: p. 24-32.
105. Wilson NS, El-Sukkari D, Belz GT, Smith CM, Steptoe RJ, Heath WR, Shortman K, and Villadangos JA, *Most lymphoid organ dendritic cell types are phenotypically and functionally immature.* Blood, 2003. **102**(6): p. 2187-94.
106. Lee HK, Zamora M, Linehan MM, Iijima N, Gonzalez D, Haberman A, and Iwasaki A, *Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection.* J Exp Med, 2009. **206**(2): p. 359-70.
107. Chiba M, Hanes J, and Langer R, *Controlled protein delivery from biodegradable tyrosine-containing poly(anhydride-co-imide) microspheres.* Biomaterials, 1997. **18**(13): p. 893-901.
108. Gupta RK and Siber GR, *Comparison of adjuvant activities of aluminium phosphate, calcium phosphate and stearyl tyrosine for tetanus toxoid.* Biologicals, 1994. **22**(1): p. 53-63.
109. Williams J, Fox-Leyva L, Christensen C, Fisher D, Schlicting E, Snowball M, Negus S, Mayers J, Koller R, and Stout R, *Hepatitis A vaccine administration: comparison between jet-injector and needle injection.* Vaccine, 2000. **18**(18): p. 1939-43.
110. Agren LC, Ekman L, Lowenadler B, and Lycke NY, *Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit.* J Immunol, 1997. **158**(8): p. 3936-46.
111. Connell TD, *Cholera toxin, LT-I, LT-IIa and LT-IIb: the critical role of ganglioside binding in immunomodulation by type I and type II heat-labile enterotoxins.* Expert Rev Vaccines, 2007. **6**(5): p. 821-34.
112. McNeela EA and Mills KH, *Manipulating the immune system: humoral versus cell-mediated immunity.* Adv Drug Deliv Rev, 2001. **51**(1-3): p. 43-54.
113. Snider DP, *The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins.* Crit Rev Immunol, 1995. **15**(3-4): p. 317-48.
114. Lycke N, *From toxin to adjuvant: the rational design of a vaccine adjuvant vector, CTA1-DD/ISCOM.* Cell Microbiol, 2004. **6**(1): p. 23-32.
115. Glenn GM, Rao M, Matyas GR, and Alving CR, *Skin immunization made possible by cholera toxin.* Nature, 1998. **391**(6670): p. 851.
116. Glenn GM, Scharton-Kersten T, Vassell R, Mallett CP, Hale TL, and Alving CR, *Transcutaneous immunization with cholera toxin protects mice against lethal mucosal toxin challenge.* J Immunol, 1998. **161**(7): p. 3211-4.
117. Scharton-Kersten T, Glenn GM, Vassell R, Yu J, Walwender D, and Alving CR, *Principles of transcutaneous immunization using cholera toxin as an adjuvant.* Vaccine, 1999. **17 Suppl 2**: p. S37-43.
118. Beignon AS, Briand JP, Muller S, and Partidos CD, *Immunization onto bare skin with heat-labile enterotoxin of Escherichia coli enhances immune responses to coadministered protein and peptide antigens and protects mice against lethal toxin challenge.* Immunology, 2001. **102**(3): p. 344-51.
119. Godefroy S, Goestch L, Plotnicky-Gilquin H, Nguyen TN, Schmitt D, Staquet MJ, and Corvaia N, *Immunization onto shaved skin with a bacterial enterotoxin adjuvant protects mice against respiratory syncytial virus (RSV).* Vaccine, 2003. **21**(15): p. 1665-71.
120. Kahlon R, Hu Y, Orteu CH, Kifayet A, Trudeau JD, Tan R, and Dutz JP, *Optimization of epicutaneous immunization for the induction of CTL.* Vaccine, 2003. **21**(21-22): p. 2890-9.

121. Scharton-Kersten T, Yu J, Vassell R, O'Hagan D, Alving CR, and Glenn GM, *Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants.* Infect Immun, 2000. **68**(9): p. 5306-13.
122. Hammond SA, Walwender D, Alving CR, and Glenn GM, *Transcutaneous immunization: T cell responses and boosting of existing immunity.* Vaccine, 2001. **19**(17-19): p. 2701-7.
123. Tierney R, Beignon AS, Rappuoli R, Muller S, Sesardic D, and Partidos CD, *Transcutaneous immunization with tetanus toxoid and mutants of Escherichia coli heat-labile enterotoxin as adjuvants elicits strong protective antibody responses.* J Infect Dis, 2003. **188**(5): p. 753-8.
124. Beignon AS, Briand JP, Rappuoli R, Muller S, and Partidos CD, *The LTR72 mutant of heat-labile enterotoxin of Escherichia coli enhances the ability of peptide antigens to elicit CD4(+) T cells and secrete gamma interferon after coapplication onto bare skin.* Infection and immunity, 2002. **70**(6): p. 3012-3019.
125. Fingerut E, Gutter B, Goldway M, Eliahoo D, and Pitcovski J, *B subunit of E. coli enterotoxin as adjuvant and carrier in oral and skin vaccination.* Vet Immunol Immunopathol, 2006. **112**(3-4): p. 253-63.
126. Ding Z, Van Riet E, Romeijn S, Kersten GF, Jiskoot W, and Bouwstra JA, *Immune modulation by adjuvants combined with diphtheria toxoid administered topically in BALB/c mice after microneedle array pretreatment.* Pharm Res, 2009. **26**(7): p. 1635-43.
127. Ding Z, Verbaan FJ, Bivas-Benita M, Bungener L, Huckriede A, van den Berg DJ, Kersten G, and Bouwstra JA, *Microneedle arrays for the transcutaneous immunization of diphtheria and influenza in BALB/c mice.* J Control Release, 2009. **136**(1): p. 71-8.
128. Stickings P, Peyre M, Coombes L, Muller S, Rappuoli R, Del Giudice G, Partidos CD, and Sesardic D, *Transcutaneous immunization with Cross-Reacting Material CRM197 of diphtheria toxin boosts functional antibody levels in mice primed parenterally with adsorbed diphtheria toxoid vaccine.* Infect Immun, 2008. **76**(4): p. 1766-1773.
129. Skountzou I, Quan FS, Jacob J, Compans RW, and Kang SM, *Transcutaneous immunization with inactivated influenza virus induces protective immune responses.* Vaccine, 2006. **24**(35-36): p. 6110-9.
130. Bal SM, Ding Z, Kersten G, Jiskoot W, and Bouwstra JA, *Microneedle-based transcutaneous immunisation in mice with N-trimethyl chitosan adjuvanted diphtheria toxoid formulations.* Pharm Res, 2010. **27**(9): p. 1837-47.
131. Beignon AS, Briand JP, Muller S, and Partidos CD, *Immunization onto bare skin with synthetic peptides: immunomodulation with a CpG-containing oligodeoxynucleotide and effective priming of influenza virus-specific CD4(+) T cells.* Immunology, 2002. **105**(2): p. 204-212.
132. Anjuere F, George-Chandy A, Audant F, Rousseau D, Holmgren J, and Czerkinsky C, *Transcutaneous immunization with cholera toxin B subunit adjuvant suppresses IgE antibody responses via selective induction of Th1 immune responses.* J Immunol, 2003. **170**(3): p. 1586-92.
133. Beignon AS, Brown F, Eftekhari P, Kramer E, Briand JP, Muller S, and Partidos CD, *A peptide vaccine administered transcutaneously together with cholera toxin elicits potent neutralising anti-FMDV antibody responses.* Vet Immunol Immunopathol, 2005. **104**(3-4): p. 273-80.
134. Verthelyi D and Zeuner RA, *Differential signaling by CpG DNA in DCs and B cells: not just TLR9.* Trends Immunol, 2003. **24**(10): p. 519-22.
135. Diwan M, Tafaghodi M, and Samuel J, *Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres.* J Control Release, 2002. **85**(1-3): p. 247-62.
136. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, and Akira S, *A Toll-like receptor recognizes bacterial DNA.* Nature, 2000. **408**(6813): p. 740-5.
137. Ozaki T, Yauchi M, Xin KQ, Hirahara F, and Okuda K, *Cross-reactive protection against influenza A virus by a topically applied DNA vaccine encoding M gene with adjuvant.* Viral Immunol, 2005. **18**(2): p. 373-80.

138. Ban E, Dupre L, Hermann E, Rohn W, Vendeville C, Quatannens B, Ricciardi-Castagnoli P, Capron A, and Riveau G, *CpG motifs induce Langerhans cell migration in vivo.* Int Immunol, 2000. **12**(6): p. 737-45.
139. Jakob T, Walker PS, Krieg AM, von Stebut E, Udey MC, and Vogel JC, *Bacterial DNA and CpG-containing oligodeoxynucleotides activate cutaneous dendritic cells and induce IL-12 production: implications for the augmentation of Th1 responses.* Int Arch Allergy Immunol, 1999. **118**(2-4): p. 457-61.
140. Chen D, Erickson CA, Endres RL, Periwal SB, Chu Q, Shu C, Maa YF, and Payne LG, *Adjuvantation of epidermal powder immunization.* Vaccine, 2001. **19**(20-22): p. 2908-17.
141. Schirmbeck R and Reimann J, *Modulation of gene-gun-mediated Th2 immunity to hepatitis B surface antigen by bacterial CpG motifs or IL-12.* Intervirology, 2001. **44**(2-3): p. 115-123.
142. Belyakov IM, Hammond SA, Ahlers JD, Glenn GM, and Berzofsky JA, *Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells.* J Clin Invest, 2004. **113**(7): p. 998-1007.
143. Halperin SA, Van Nest G, Smith B, Abtahi S, Whiley H, and Eiden JJ, *A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant.* Vaccine, 2003. **21**(19-20): p. 2461-7.
144. Cooper CL, Davis HL, Morris ML, Efler SM, Adhami MA, Krieg AM, Cameron DW, and Heathcote J, *CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind phase I/II study.* J Clin Immunol, 2004. **24**(6): p. 693-701.
145. Vandepapeliere P, Horsmans Y, Moris P, Van Mechelen M, Janssens M, Koutsoukos M, Van Belle P, Clement F, Hanon E, Wettendorff M, Garcon N, and Leroux-Roels G, *Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers.* Vaccine, 2008. **26**(10): p. 1375-86.
146. Glenn GM, Scharton-Kersten T, and Alving CR, *Advances in vaccine delivery: transcutaneous immunisation.* Expert Opin Investig Drugs, 1999. **8**(6): p. 797-805.
147. Beignon AS, Briand JP, Rappuoli R, Muller S, and Partidos CD, *The LTR72 mutant of heat-labile enterotoxin of Escherichia coli enhances the ability of peptide antigens to elicit CD4(+) T cells and secrete gamma interferon after coapplication onto bare skin.* Infect Immun, 2002. **70**(6): p. 3012-9.
148. Najar HM and Dutz JP, *Topical TLR9 agonists induce more efficient cross-presentation of injected protein antigen than parenteral TLR9 agonists do.* Eur J Immunol, 2007. **37**(8): p. 2242-56.
149. Inoue J and Aramaki Y, *Toll-like receptor-9 expression induced by tape-stripping triggers on effective immune response with CpG-oligodeoxynucleotides.* Vaccine, 2007. **25**(6): p. 1007-13.
150. Rechtsteiner G, Warger T, Osterloh P, Schild H, and Radsak MP, *Cutting edge: priming of CTL by transcutaneous peptide immunization with imiquimod.* J Immunol, 2005. **174**(5): p. 2476-80.
151. Chang BA, Cross JL, Najar HM, and Dutz JP, *Topical resiquimod promotes priming of CTL to parenteral antigens.* Vaccine, 2009. **27**(42): p. 5791-5799.
152. Wood LC, Jackson SM, Elias PM, Grunfeld C, and Feingold KR, *Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice.* J Clin Invest, 1992. **90**(2): p. 482-7.
153. Cumberbatch M, Dearman RJ, and Kimber I, *Langerhans cells require signals from both tumour necrosis factor-alpha and interleukin-1 beta for migration.* Immunology, 1997. **92**(3): p. 388-95.
154. Donnelly RF, Raj Singh TR, and Woolfson AD, *Microneedle-based drug delivery systems: Microfabrication, drug delivery, and safety.* Drug Deliv, 2010. **17**(4): p. 187-207.
155. Weniger BG and Papania MJ, *Alternative vaccine delivery methods*, in *Vaccines*, Plotkin S.A., Orenstein W.A., and Offit P.A., Editors. 2008, Elsevier: Amsterdam. p. 1357-1392.
156. Van Damme P, Oosterhuis-Kafeja F, Van der Wielen M, Almagor Y, Sharon O, and Levin Y, *Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults.* Vaccine, 2009. **27**(3): p. 454-9.
157. Kenney RT, Frech SA, Muenz LR, Villar CP, and Glenn GM, *Dose sparing with intradermal injection of influenza vaccine.* N Engl J Med, 2004. **351**(22): p. 2295-301.

158. Laurent PE, Bonnet S, Alchas P, Regolini P, Mikszta JA, Pettis R, and Harvey NG, *Evaluation of the clinical performance of a new intradermal vaccine administration technique and associated delivery system.* Vaccine, 2007. **25**(52): p. 8833-42.
159. Beran J, Ambrozaitis A, Laiskonis A, Mickuviene N, Bacart P, Calozet Y, Demanet E, Heijmans S, Van Belle P, Weber F, and Salamand C, *Intradermal influenza vaccination of healthy adults using a new microinjection system: a 3-year randomised controlled safety and immunogenicity trial.* BMC Med, 2009. **7**: p. 13-28.
160. Gerstel MS and Place VA, *Drug delivery device*. 1976: US patent No. 3,964,482.
161. Haut RC, *Biomechanics of soft tissue*. 2nd ed. 2002, New York: Springer.
162. Reihsner R, Balogh B, and Menzel EJ, *Two-dimensional elastic properties of human skin in terms of an incremental model at the in vivo configuration.* Med Eng Phys, 1995. **17**(4): p. 304-13.
163. Widera G, Johnson J, Kim L, Libiran L, Nyam K, Daddona PE, and Cormier M, *Effect of delivery parameters on immunization to ovalbumin following intracutaneous administration by a coated microneedle array patch system.* Vaccine, 2006. **24**(10): p. 1653-64.
164. Martanto W, Moore JS, Kashlan O, Kamath R, Wang PM, O'Neal JM, and Prausnitz MR, *Microinfusion using hollow microneedles.* Pharm Res, 2006. **23**(1): p. 104-13.
165. Martanto W, Moore JS, Couse T, and Prausnitz MR, *Mechanism of fluid infusion during microneedle insertion and retraction.* J Control Release, 2006. **112**(3): p. 357-61.
166. Davis SP, Landis BJ, Adams ZH, Allen MG, and Prausnitz MR, *Insertion of microneedles into skin: measurement and prediction of insertion force and needle fracture force.* J Biomech, 2004. **37**(8): p. 1155-63.
167. Prausnitz MR and Langer R, *Transdermal drug delivery.* Nat Biotechnol, 2008. **26**(11): p. 1261-8.
168. Prausnitz MR, Mikszta JA, Cormier M, and Andrianov AK, *Microneedle-based vaccines.* Curr Top Microbiol Immunol, 2009. **333**: p. 369-93.
169. Henry S, McAllister DV, Allen MG, and Prausnitz MR, *Microfabricated microneedles: A novel approach to transdermal drug delivery.* J Pharm Sci, 1998. **87**(8): p. 922-5.
170. Banks SL, Pinninti RR, Gill HS, Crooks PA, Prausnitz MR, and Stinchcomb AL, *Flux across microneedle-treated skin is increased by increasing charge of naltrexone and naltrexol in vitro.* Pharm Res, 2008. **25**(7): p. 1677-85.
171. Verbaan FJ, Bal SM, van den Berg DJ, Dijksman JA, van Hecke M, Verpoorten H, van den Berg A, Luttge R, and Bouwstra JA, *Improved piercing of microneedle arrays in dermatomed human skin by an impact insertion method.* J Control Release, 2008. **128**(1): p. 80-8.
172. Ding Z, Bal SM, Romeijn S, Kersten GF, Jiskoot W, and Bouwstra JA, *Transcutaneous immunization studies in mice using diphtheria toxoid-loaded vesicle formulations and a microneedle array.* Pharm Res, 2010.
173. Donnelly RF, Morrow DI, McCarron PA, Woolfson AD, Morrissey A, Juzenas P, Juzeniene A, Iani V, McCarthy HO, and Moan J, *Microneedle-mediated intradermal delivery of 5-aminolevulinic acid: potential for enhanced topical photodynamic therapy.* J Control Release, 2008. **129**(3): p. 154-62.
174. Badran MM, Kuntsche J, and Fahr A, *Skin penetration enhancement by a microneedle device (Dermaroller) in vitro: dependency on needle size and applied formulation.* Eur J Pharm Sci, 2009. **36**(4-5): p. 511-23.
175. *The 3M Microstructured Transdermal System*. 2009 [cited June 15th, 2010; Available from: http://solutions.3m.com/3MContentRetrievalAPI/BlobServlet?locale=en_WW&lmd=1262009493000&assetId=1258559455435&assetType=MMM_Image&blobAttribute=ImageFile.
176. Matriano JA, Cormier M, Johnson J, Young WA, Buttery M, Nyam K, and Daddona PE, *Macroflux microprojection array patch technology: a new and efficient approach for intracutaneous immunization.* Pharm Res, 2002. **19**(1): p. 63-70.
177. Gill HS and Prausnitz MR, *Coated microneedles for transdermal delivery.* J Control Release, 2007. **117**(2): p. 227-37.
178. Zhu Q, Zarnitsyn VG, Ye L, Wen Z, Gao Y, Pan L, Skountzou I, Gill HS, Prausnitz MR, Yang C, and Compans RW, *Immunization by vaccine-coated microneedle arrays protects against lethal influenza virus challenge.* Proc Natl Acad Sci U S A, 2009. **106**(19): p. 7968-73.

179. Gill HS, Soderholm J, Prausnitz MR, and Sallberg M, *Cutaneous vaccination using microneedles coated with hepatitis C DNA vaccine.* Gene Ther, 2010: p. 811-814.
180. Koutsonanos DG, del Pilar Martin M, Zarnitsyn VG, Sullivan SP, Compans RW, Prausnitz MR, and Skountzou I, *Transdermal influenza immunization with vaccine-coated microneedle arrays.* PLoS ONE, 2009. **4**(3): p. e4773.
181. Chen X, Prow TW, Crichton ML, Jenkins DW, Roberts MS, Frazer IH, Fernando GJ, and Kendall MA, *Dry-coated microprojection array patches for targeted delivery of immunotherapeutics to the skin.* J Control Release, 2009. **139**(3): p. 212-20.
182. Fernando GJ, Chen X, Prow TW, Crichton ML, Fairmaid EJ, Roberts MS, Frazer IH, Brown LE, and Kendall MA, *Potent immunity to low doses of influenza vaccine by probabilistic guided micro-targeted skin delivery in a mouse model.* PLoS ONE, 2010. **5**(4): p. e10266.
183. Crichton ML, Ansaldo A, Chen X, Prow TW, Fernando GJ, and Kendall MA, *The effect of strain rate on the precision of penetration of short densely-packed microprojection array patches coated with vaccine.* Biomaterials, 2010. **31**(16): p. 4562-72.
184. Kim YC, Quan FS, Yoo DG, Compans RW, Kang SM, and Prausnitz MR, *Improved influenza vaccination in the skin using vaccine coated microneedles.* Vaccine, 2009. **27**(49): p. 6932-8.
185. Kim YC, Quan FS, Compans RW, Kang SM, and Prausnitz MR, *Stability kinetics of influenza vaccine coated onto microneedles during drying and storage.* Pharm Res, 2010.
186. Gill HS and Prausnitz MR, *Coating formulations for microneedles.* Pharm Res, 2007. **24**(7): p. 1369-80.
187. Kim YC, Quan FS, Compans RW, Kang SM, and Prausnitz MR, *Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity.* J Control Release, 2009: p. 187-195.
188. Griss P and Stemme G, *Side-opened out-of-plane microneedles for microfluidic transdermal liquid transfer.* J Microelectromech Syst, 2003. **12**(3): p. 296-301.
189. Wang PM, Cornwell M, Hill J, and Prausnitz MR, *Precise microinjection into skin using hollow microneedles.* J Invest Dermatol, 2006. **126**(5): p. 1080-7.
190. McAllsiter DV, Cros F, Davis SP, Matta LM, Prausnitz MR, and Allen MG, *Three-dimensional hollow microneedles and microtube arrays*, in *Transducers'99. The 10th Int. Conf. on Solid-state Sensors and Actuators*. 1999: Sendai, Japan. p. 1098-1101.
191. Hafeli UO, Mokhtari A, Liepmann D, and Stoeber B, *In vivo evaluation of a microneedle-based miniature syringe for intradermal drug delivery.* Biomed Microdevices, 2009.
192. *BD Soluvia™ prefillable microinjection system*. [cited June 15th, 2010; Available from: http://www.bd.com/pharmaceuticals/products/microinjection.asp.
193. *Dissolving Microneedles* 2009 [cited June 15th, 2010; Available from: http://www.bioserentach.co.jp/e-detail-microneedle.html.
194. Mikszta JA, Alarcon JB, Brittingham JM, Sutter DE, Pettis RJ, and Harvey NG, *Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery.* Nat Med, 2002. **8**(4): p. 415-9.
195. *Intercell's needle-free patches*. 2008 [cited June 15th, 2010; Available from: http://www.fastcompany.com/magazine/130/intercell.html.
196. *Five New Needle-Free Delivery Devices*. 2007 [cited June 15th, 2010; Available from: http://www.onemedplace.com/blog/archives/362.
197. *How does PMED™ work?* 2006 [cited June 15th, 2010; Available from: http://web.archive.org/web/20061118180018/www.powdermed.com/developmentPmed.htm.
198. Miyano T, Tobinaga Y, Kanno T, Matsuzaki Y, Takeda H, Wakui M, and Hanada K, *Sugar micro needles as transdermic drug delivery system.* Biomed Microdevices, 2005. **7**(3): p. 185-8.
199. Ito Y, Hagiwara E, Saeki A, Sugioka N, and Takada K, *Feasibility of microneedles for percutaneous absorption of insulin.* Eur J Pharm Sci, 2006. **29**(1): p. 82-8.
200. Ito Y, Yoshimitsu J, Shiroyama K, Sugioka N, and Takada K, *Self-dissolving microneedles for the percutaneous absorption of EPO in mice.* J Drug Target, 2006. **14**(5): p. 255-61.

201. Park JH, Allen MG, and Prausnitz MR, *Polymer microneedles for controlled-release drug delivery.* Pharm Res, 2006. **23**(5): p. 1008-19.
202. Kolli CS and Banga AK, *Characterization of solid maltose microneedles and their use for transdermal delivery.* Pharm Res, 2008. **25**(1): p. 104-13.
203. Lee JW, Park JH, and Prausnitz MR, *Dissolving microneedles for transdermal drug delivery.* Biomaterials, 2008. **29**(13): p. 2113-24.
204. Li G, Badkar A, Nema S, Kolli CS, and Banga AK, *In vitro transdermal delivery of therapeutic antibodies using maltose microneedles.* Int J Pharm, 2009. **368**(1-2): p. 109-15.
205. Donnelly RF, Morrow DI, Singh TR, Migalska K, McCarron PA, O'Mahony C, and Woolfson AD, *Processing difficulties and instability of carbohydrate microneedle arrays.* Drug Dev Ind Pharm, 2009. **35**(10): p. 1242-54.
206. Sullivan SP, Koutsonanos DG, Del Pilar Martin M, Lee JW, Zarnitsyn V, Choi SO, Murthy N, Compans RW, Skountzou I, and Prausnitz MR, *Dissolving polymer microneedle patches for influenza vaccination.* Nat Med. **16**(8): p. 915-20.
207. Sullivan SP, Murthy N, and Prausnitz MR, *Minimally invasive protein delivery with rapidly dissolving polymer microneedles.* Adv. Mater., 2008. **20**(5): p. 933-938.
208. *Emerging microneedles: Manufacturers race to be first to market with microneedle transdermal patches*. 2008 [cited June 15th, 2010; Available from: http://www.micromanufacturing.com/showthread.php?p=555.
209. Hooper JW, Golden JW, Ferro AM, and King AD, *Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge.* Vaccine, 2007. **25**(10): p. 1814-23.
210. Verbaan FJ, Bal SM, van den Berg DJ, Groenink WH, Verpoorten H, Luttge R, and Bouwstra JA, *Assembled microneedle arrays enhance the transport of compounds varying over a large range of molecular weight across human dermatomed skin.* J Control Release, 2007. **117**(2): p. 238-45.
211. Aggarwal G, Garg A, and Dhawan S, *Transdermal drug delivery: evolving technologies and expanding opportunities.* Indian J. Pharm. Educ. Res., 2009. **43**(3): p. 251-259.
212. Arora A, Prausnitz MR, and Mitragotri S, *Micro-scale devices for transdermal drug delivery.* Int J Pharm, 2008. **364**(2): p. 227-36.
213. Glenn GM, Villar CP, Flyer DC, Bourgeois AL, McKenzie R, Lavker RM, and Frech SA, *Safety and immunogenicity of an enterotoxigenic Escherichia coli vaccine patch containing heat-labile toxin: use of skin pretreatment to disrupt the stratum corneum.* Infect Immun, 2007. **75**(5): p. 2163-70.
214. McKenzie R, Bourgeois AL, Frech SA, Flyer DC, Bloom A, Kazempour K, and Glenn GM, *Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic Escherichia coli (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study.* Vaccine, 2007. **25**(18): p. 3684-91.
215. Glenn GM, Taylor DN, Li X, Frankel S, Montemarano A, and Alving CR, *Transcutaneous immunization: a human vaccine delivery strategy using a patch.* Nat Med, 2000. **6**(12): p. 1403-6.
216. Frech SA, DuPont HL, Bourgeois AL, McKenzie R, Belkind-Gerson J, Figueroa JF, Okhuysen PC, Guerrero NH, Martinez-Sandoval FG, Melendez-Romero JHM, Jiang ZD, Asturias EJ, Halpern J, Torres OR, Hoffman AS, Villar CP, Kassem RN, Flyer DC, Andersen BH, Kazempour K, Breisch SA, and Glenn GM, *Use of a patch containing heat-labile toxin from Escherichia coli against travellers' diarrhoea: A phase II, randomised, double-blind, placebo-controlled field trial.* Lancet, 2008. **371**(9629): p. 2019-2025.
217. Bouwstra JA, de Graaff A, Gooris GS, Nijsse J, Wiechers JW, and van Aelst AC, *Water distribution and related morphology in human stratum corneum at different hydration levels.* J Invest Dermatol, 2003. **120**(5): p. 750-8.
218. Garg S, Hoelscher M, Belser JA, Wang C, Jayashankar L, Guo Z, Durland RH, Katz JM, and Sambhara S, *Needle-free skin patch delivery of a vaccine for a potentially pandemic influenza virus provides protection against lethal challenge in mice.* Clin Vaccine Immunol, 2007. **14**(7): p. 926-8.
219. Tezel A, Paliwal S, Shen Z, and Mitragotri S, *Low-frequency ultrasound as a transcutaneous immunization adjuvant.* Vaccine, 2005. **23**(29): p. 3800-7.

220. Zhao YL, Murthy SN, Manjili MH, Guan LJ, Sen A, and Hui SW, *Induction of cytotoxic T-lymphocytes by electroporation-enhanced needle-free skin immunization.* Vaccine, 2006. **24**(9): p. 1282-90.
221. Chen D, Weis KF, Chu Q, Erickson C, Endres R, Lively CR, Osorio J, and Payne LG, *Epidermal powder immunization induces both cytotoxic T-lymphocyte and antibody responses to protein antigens of influenza and hepatitis B viruses.* J Virol, 2001. **75**(23): p. 11630-40.
222. Chen D, Zuleger C, Chu Q, Maa YF, Osorio J, and Payne LG, *Epidermal powder immunization with a recombinant HIV gp120 targets Langerhans cells and induces enhanced immune responses.* AIDS Res Hum Retroviruses, 2002. **18**(10): p. 715-22.
223. Chen D, Endres R, Maa YF, Kensil CR, Whitaker-Dowling P, Trichel A, Youngner JS, and Payne LG, *Epidermal powder immunization of mice and monkeys with an influenza vaccine.* Vaccine, 2003. **21**(21-22): p. 2830-6.
224. Roberts LK, Barr LJ, Fuller DH, McMahon CW, Leese PT, and Jones S, *Clinical safety and efficacy of a powdered Hepatitis B nucleic acid vaccine delivered to the epidermis by a commercial prototype device.* Vaccine, 2005. **23**(40): p. 4867-78.
225. Drape RJ, Macklin MD, Barr LJ, Jones S, Haynes JR, and Dean HJ, *Epidermal DNA vaccine for influenza is immunogenic in humans.* Vaccine, 2006. **24**(21): p. 4475-81.
226. Dincer Z, Jones S, and Haworth R, *Preclinical safety assessment of a DNA vaccine using particle-mediated epidermal delivery in domestic pig, minipig and mouse.* Exp Toxicol Pathol, 2006. **57**(5-6): p. 351-7.
227. Cassaday RD, Sondel PM, King DM, Macklin MD, Gan J, Warner TF, Zuleger CL, Bridges AJ, Schalch HG, Kim KM, Hank JA, Mahvi DM, and Albertini MR, *A phase I study of immunization using particle-mediated epidermal delivery of genes for gp100 and GM-CSF into uninvolved skin of melanoma patients.* Clin Cancer Res, 2007. **13**(2 Pt 1): p. 540-9.
228. Braun RP, Dong L, Jerome S, Herber R, Roberts LK, and Payne LG, *Multi-antigenic DNA immunization using herpes simplex virus type 2 genomic fragments.* Hum Vaccin, 2008. **4**(1): p. 36-43.
229. Jones S, Evans K, McElwaine-Johnn H, Sharpe M, Oxford J, Lambkin-Williams R, Mant T, Nolan A, Zambon M, Ellis J, Beadle J, and Loudon PT, *DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial.* Vaccine, 2009. **27**(18): p. 2506-12.
230. Canter J, Mackey K, Good LS, Roberto RR, Chin J, Bond WW, Alter MJ, and Horan JM, *An outbreak of hepatitis B associated with jet injections in a weight reduction clinic.* Arch Intern Med, 1990. **150**(9): p. 1923-7.
231. Mitragotri S, *Immunization without needles.* Nat Rev Immunol, 2005. **5**(12): p. 905-16.
232. Mikszta JA, Dekker JP, 3rd, Harvey NG, Dean CH, Brittingham JM, Huang J, Sullivan VJ, Dyas B, Roy CJ, and Ulrich RG, *Microneedle-based intradermal delivery of the anthrax recombinant protective antigen vaccine.* Infect Immun, 2006. **74**(12): p. 6806-10.
233. Glenn GM, Flyer DC, Ellingsworth LR, Frech SA, Frerichs DM, Seid RC, and Yu J, *Transcutaneous immunization with heat-labile enterotoxin: development of a needle-free vaccine patch.* Expert Rev Vaccines, 2007. **6**(5): p. 809-19.
234. *Travelers' diarrhea vaccine patch*. 2009 [cited June 15th, 2010; Available from: http://www.intercell.com/main/forbeginners/news/not-in-menu/news-full/back_to/travelers-diarrhea-vaccine-patch/article/intercell-provides-update-on-phase-iii-trial-for-travelers-diarrhea-vaccine-patch/.
235. Frech SA, Kenney RT, Spyr CA, Lazar H, Viret JF, Herzog C, Gluck R, and Glenn GM, *Improved immune responses to influenza vaccination in the elderly using an immunostimulant patch.* Vaccine, 2005. **23**(7): p. 946-50.
236. Matyas GR, Friedlander AM, Glenn GM, Little S, Yu J, and Alving CR, *Needle-free skin patch vaccination method for anthrax.* Infect Immun, 2004. **72**(2): p. 1181-3.
237. Combadiere B, Vogt A, Mahe B, Costagliola D, Hadam S, Bonduelle O, Sterry W, Staszewski S, Schaefer H, van der Werf S, Katlama C, Autran B, and Blume-Peytavi U, *Preferential amplification of CD8 effector-T cells after transcutaneous application of an inactivated influenza vaccine: a randomized phase I trial.* PLoS ONE, 2010. **5**(5): p. e10818.

238. Vogt A, Mahe B, Costagliola D, Bonduelle O, Hadam S, Schaefer G, Schaefer H, Katlama C, Sterry W, Autran B, Blume-Peytavi U, and Combadiere B, *Transcutaneous anti-influenza vaccination promotes both CD4 and CD8 T cell immune responses in humans.* J Immunol, 2008. **180**(3): p. 1482-9.
239. Yagi H, Hashizume H, Horibe T, Yoshinari Y, Hata M, Ohshima A, Ito T, Takigawa M, Shibaki A, Shimizu H, and Seo N, *Induction of therapeutically relevant cytotoxic T lymphocytes in humans by percutaneous peptide immunization.* Cancer Res, 2006. **66**(20): p. 10136-44.
240. Mahe B, Vogt A, Liard C, Duffy D, Abadie V, Bonduelle O, Boissonnas A, Sterry W, Verrier B, Blume-Peytavi U, and Combadiere B, *Nanoparticle-based targeting of vaccine compounds to skin antigen-presenting cells by hair follicles and their transport in mice.* J Invest Dermatol, 2009. **129**(5): p. 1156-64.
241. Cristillo AD, Weiss D, Hudacik L, Restrepo S, Galmin L, Suschak J, Draghia-Akli R, Markham P, and Pal R, *Persistent antibody and T cell responses induced by HIV-1 DNA vaccine delivered by electroporation.* Biochem Biophys Res Commun, 2008. **366**(1): p. 29-35.
242. Hirao LA, Wu L, Khan AS, Satishchandran A, Draghia-Akli R, and Weiner DB, *Intradermal/subcutaneous immunization by electroporation improves plasmid vaccine delivery and potency in pigs and rhesus macaques.* Vaccine, 2008. **26**(3): p. 440-8.
243. Vandermeulen G, Daugimont L, Richiardi H, Vanderhaeghen ML, Lecouturier N, Ucakar B, and Preat V, *Effect of tape stripping and adjuvants on immune response after intradermal DNA electroporation.* Pharm Res, 2009. **26**(7): p. 1745-51.
244. Wang R, Epstein J, Baraceros FM, Gorak EJ, Charoenvit Y, Carucci DJ, Hedstrom RC, Rahardjo N, Gay T, Hobart P, Stout R, Jones TR, Richie TL, Parker SE, Doolan DL, Norman J, and Hoffman SL, *Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10817-22.
245. Epstein JE, Gorak EJ, Charoenvit Y, Wang R, Freydberg N, Osinowo O, Richie TL, Stoltz EL, Trespalacios F, Nerges J, Ng J, Fallarme-Majam V, Abot E, Goh L, Parker S, Kumar S, Hedstrom RC, Norman J, Stout R, and Hoffman SL, *Safety, tolerability, and lack of antibody responses after administration of a PfCSP DNA malaria vaccine via needle or needle-free jet injection, and comparison of intramuscular and combination intramuscular/intradermal routes.* Hum Gene Ther, 2002. **13**(13): p. 1551-60.
246. Aboud S, Nilsson C, Karlen K, Marovich M, Wahren B, Sandstrom E, Gaines H, Biberfeld G, and Godoy-Ramirez K, *Strong HIV-Specific CD4+ and CD8+ T lymphocyte proliferative responses in healthy individuals immunized with a HIV-1 DNA vaccine and boosted with HIV-1 recombinant Modified Vaccinia virus Ankara (MVA).* Clin Vaccine Immunol, 2010.
247. Raviprakash K, Ewing D, Simmons M, Porter KR, Jones TR, Hayes CG, Stout R, and Murphy GS, *Needle-free Biojector injection of a dengue virus type 1 DNA vaccine with human immunostimulatory sequences and the GM-CSF gene increases immunogenicity and protection from virus challenge in Aotus monkeys.* Virology, 2003. **315**(2): p. 345-52.
248. Choi AH, Smiley K, Basu M, McNeal MM, Shao M, Bean JA, Clements JD, Stout RR, and Ward RL, *Protection of mice against rotavirus challenge following intradermal DNA immunization by Biojector needle-free injection.* Vaccine, 2007. **25**(16): p. 3215-8.
249. Babiuk S, Baca-Estrada M, Babiuk LA, Ewen C, and Foldvari M, *Cutaneous vaccination: the skin as an immunologically active tissue and the challenge of antigen delivery.* J Control Release, 2000. **66**(2-3): p. 199-214.
250. Combadiere B and Mahe B, *Particle-based vaccines for transcutaneous vaccination.* Comp Immunol Microbiol Infect Dis, 2008. **31**(2-3): p. 293-315.
251. Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, and Bramwell VW, *Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens.* Int J Pharm, 2008. **364**(2): p. 272-80.
252. Schlosser E, Mueller M, Fischer S, Basta S, Busch DH, Gander B, and Groettrup M, *TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses.* Vaccine, 2008. **26**(13): p. 1626-37.

253. Korsholm KS, Agger EM, Foged C, Christensen D, Dietrich J, Andersen CS, Geisler C, and Andersen P, *The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes.* Immunology, 2007. **121**(2): p. 216-26.
254. Yan W, Chen W, and Huang L, *Mechanism of adjuvant activity of cationic liposome: phosphorylation of a MAP kinase, ERK and induction of chemokines.* Mol Immunol, 2007. **44**(15): p. 3672-81.
255. El Maghraby GM, Williams AC, and Barry BW, *Interactions of surfactants (edge activators) and skin penetration enhancers with liposomes.* Int J Pharm, 2004. **276**(1-2): p. 143-61.
256. Cevc G, Schatzlein A, Gebauer D, and Blume G, *Ultra-high efficiency of drug and peptide transfer through the intact skin by means of novel drug-carriers, transfersomes.*, in *Prediction of percutaneous penetration, 3rd International Conference* Brain K.R., James V.A., and Walters K.A., Editors. 1993, STS Publishing. p. 226-236.
257. Cevc G and Gebauer D, *Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier.* Biophys J, 2003. **84**(2 Pt 1): p. 1010-24.
258. Benson HA, *Transfersomes for transdermal drug delivery.* Expert Opin Drug Deliv, 2006. **3**(6): p. 727-37.
259. El Maghraby GM, Williams AC, and Barry BW, *Skin delivery of oestradiol from deformable and traditional liposomes: mechanistic studies.* J Pharm Pharmacol, 1999. **51**(10): p. 1123-34.
260. Jain S, Jain P, Umamaheshwari RB, and Jain NK, *Transfersomes--a novel vesicular carrier for enhanced transdermal delivery: development, characterization, and performance evaluation.* Drug Dev Ind Pharm, 2003. **29**(9): p. 1013-26.
261. El Maghraby GM, Williams AC, and Barry BW, *Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro.* J Pharm Pharmacol, 2001. **53**(8): p. 1069-77.
262. Paul A, Cevc G, and Bachhawat BK, *Transdermal immunization with large proteins by means of ultradeformable drug carriers.* Eur J Immunol, 1995. **25**(12): p. 3521-4.
263. Paul A, Cevc G, and Bachhawat BK, *Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes.* Vaccine, 1998. **16**(2-3): p. 188-95.
264. Gupta PN, Mishra V, Rawat A, Dubey P, Mahor S, Jain S, Chatterji DP, and Vyas SP, *Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study.* Int J Pharm, 2005. **293**(1-2): p. 73-82.
265. Mishra D, Dubey V, Asthana A, Saraf DK, and Jain NK, *Elastic liposomes mediated transcutaneous immunization against Hepatitis B.* Vaccine, 2006. **24**: p. 4847-4855.
266. van den Bergh BA, Frederik PMF, Bomans PHH, Junginger HE, and Bouwstra JA, *Elastic liquid state vesicles as a tool for topical drug delivery*, in *Division of Pharmceutical Technology*. 1999, Leiden University: The Netherlands. p. 49-63.
267. Bouwstra JA, Honeywell-Nguyen PL, Gooris GS, and Ponec M, *Structure of the skin barrier and its modulation by vesicular formulations.* Prog Lipid Res, 2003. **42**(1): p. 1-36.
268. Honeywell-Nguyen PL, Arenja S, and Bouwstra JA, *Skin penetration and mechanisms of action in the delivery of the D2-agonist rotigotine from surfactant-based elastic vesicle formulations.* Pharm Res, 2003. **20**(10): p. 1619-25.
269. Honeywell-Nguyen PL, Van den Bussche MH, Junginger HE, and Bouwstra JA, *The effect of surfactant-based elastic and rigid vesicles on the penetration of lidocaine across human skin.* STP Pharma, 2002. **12**: p. 257-62.
270. Honeywell-Nguyen PL, Gooris GS, and Bouwstra JA, *Quantitative assessment of the transport of elastic and rigid vesicle components and a model drug from these vesicle formulations into human skin in vivo.* J Invest Dermatol, 2004. **123**(5): p. 902-10.
271. Mishra D, Mishra PK, Dubey V, Nahar M, Dabadghao S, and Jain NK, *Systemic and mucosal immune response induced by transcutaneous immunization using Hepatitis B surface antigen-loaded modified liposomes.* Eur J Pharm Sci, 2008. **33**(4-5): p. 424-33.
272. Jain S and Vyas SP, *Mannosylated niosomes as carrier adjuvant system for topical immunization.* J Pharm Pharmacol, 2005. **57**(9): p. 1177-84.

273. Mattheolabakis G, Lagoumintzis G, Panagi Z, Papadimitriou E, Partidos CD, and Avgoustakis K, *Transcutaneous delivery of a nanoencapsulated antigen: Induction of immune responses.* Int J Pharm, 2010. **385**(1-2): p. 187-193.
274. Young SL, Wilson M, Wilson S, Beagley KW, Ward V, and Baird MA, *Transcutaneous vaccination with virus-like particles.* Vaccine, 2006. **24**(26): p. 5406-12.
275. Slutter B, Soema PC, Ding Z, Verheul R, Hennink W, and Jiskoot W, *Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen.* J Control Release, 2010. **143**(2): p. 207-14.
276. Wu Y, Przysiecki C, Flanagan E, Bello-Irizarry SN, Ionescu R, Muratova O, Dobrescu G, Lambert L, Keister D, Rippeon Y, Long CA, Shi L, Caulfield M, Shaw A, Saul A, Shiver J, and Miller LH, *Sustained high-titer antibody responses induced by conjugating a malarial vaccine candidate to outer-membrane protein complex.* Proc Natl Acad Sci U S A, 2006. **103**(48): p. 18243-8.
277. McCormick AA, Corbo TA, Wykoff-Clary S, Palmer KE, and Pogue GP, *Chemical conjugate TMV-peptide bivalent fusion vaccines improve cellular immunity and tumor protection.* Bioconjug Chem, 2006. **17**(5): p. 1330-8.
278. Bal SM, Slutter B, van Riet E, Kruithof AC, Ding Z, Kersten GF, Jiskoot W, and Bouwstra JA, *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* J Control Release, 2010. **142**(3): p. 374-83.
279. Slutter B, Plapied L, Fievez V, Sande MA, des Rieux A, Schneider YJ, Van Riet E, Jiskoot W, and Preat V, *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J Control Release, 2009. **138**(2): p. 113-21.
280. Mulholland WJ, Arbuthnott EA, Bellhouse BJ, Cornhill JF, Austyn JM, Kendall MA, Cui Z, and Tirlapur UK, *Multiphoton high-resolution 3D imaging of Langerhans cells and keratinocytes in the mouse skin model adopted for epidermal powdered immunization.* J Invest Dermatol, 2006. **126**(7): p. 1541-8.
281. Friedmann PS, *Disappearance of epidermal Langerhans cells during PUVA therapy.* Br J Dermatol, 1981. **105**(2): p. 219-21.
282. Falstie-Jensen N, Spaun E, Brochner-Mortensen J, and Falstie-Jensen S, *The influence of epidermal thickness on transcutaneous oxygen pressure measurements in normal persons.* Scand J Clin Lab Invest, 1988. **48**(6): p. 519-23.
283. Azzi L, El-Alfy M, Martel C, and Labrie F, *Gender differences in mouse skin morphology and specific effects of sex steroids and dehydroepiandrosterone.* J Investig Dermatol, 2004. **124**(1): p. 22-27.
284. Lauer AC, Lieb LM, Ramachandran C, Flynn GL, and Weiner ND, *Transfollicular drug delivery.* Pharm Res, 1995. **12**(2): p. 179-86.