Transcutaneous subunit vaccine delivery. A combined approach of vesicle formulations and microneedle arrays

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Chapter 1

Transcutaneous immunization:

A general introduction

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1. Introduction

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

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

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

Table I. Terms used in this chapter

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

2. Vaccines and adjuvants

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

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

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

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

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

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

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

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

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

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

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

3. Immunological function of the skin

3.1. Skin structure

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

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

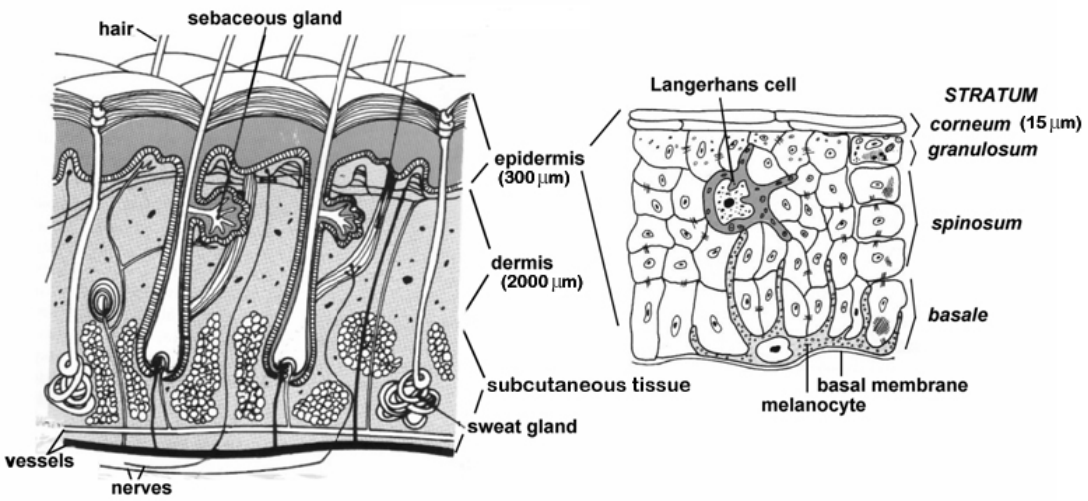

Figure 1. Structure of the skin. The skin consists of three main layers: epidermis, dermis, and subcutaneous fat tissue. The barrier function of the skin is located in the uppermost layer, the *stratum corneum*. LCs in the epidermis and the dermal DCs in the dermis are the main APCs in the skin and the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

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

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

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

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

3.2. Innate immunity

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

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

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

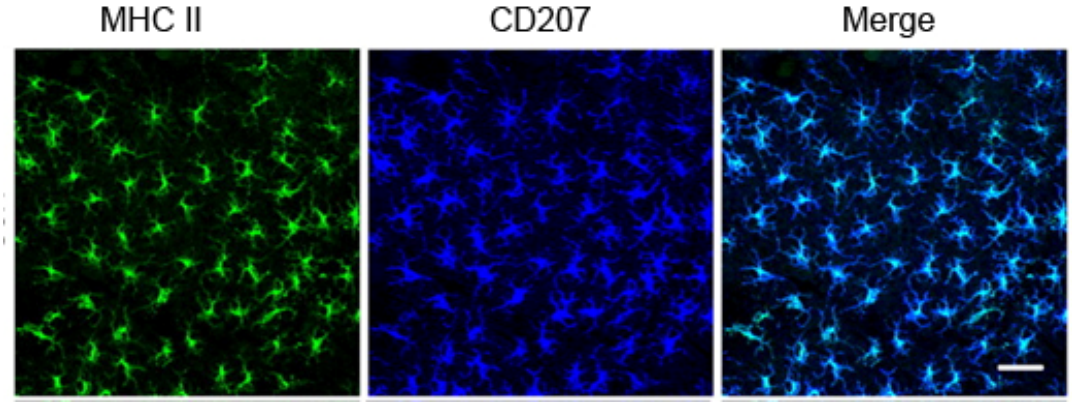

Figure 2. Epidermal LCs. Immune fluorescence staining of MHC II (green) and langerin/CD207 (blue) in epidermal sheets freshly isolated from the ear skin of a normal adult mouse (C3H, H2k). Confocal images show that all MHC II^+ cells in the epidermis express langerin. Picture adapted from Erikson *et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

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

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

3.3. Pattern-recognition receptors

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

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

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

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

*endogenous danger signals, DAMPs

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

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

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

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

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

3.4. Adaptive immunity

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

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

3.4.1. B cells

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

3.4.2. $CD4^+$ T cells

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

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

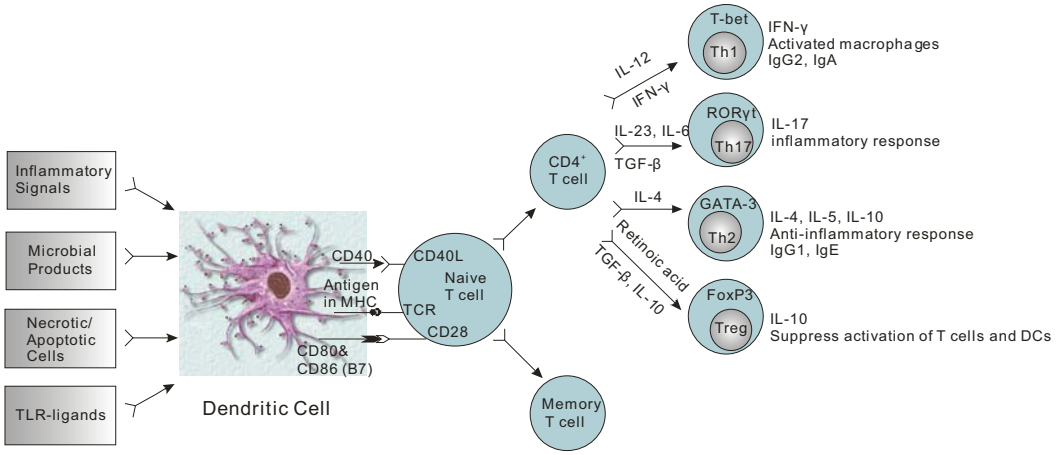

Figure 3. The adaptive immunity controlled by DCs. The specific pathway followed by $CD4^+$ T cells, whether it involves Th1, Th2, Th17 or Treg cell differentiation, is significantly governed by DCs and depending on the nature of the invading pathogens.

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

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

3.4.3. $CD8^+$ T cells

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

3.4.4. Memory cells

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

3.4.5. Skin DCs in adaptive immunity

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

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

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

4. Transcutaneous immunization

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

4.1. Overcoming the skin barrier

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

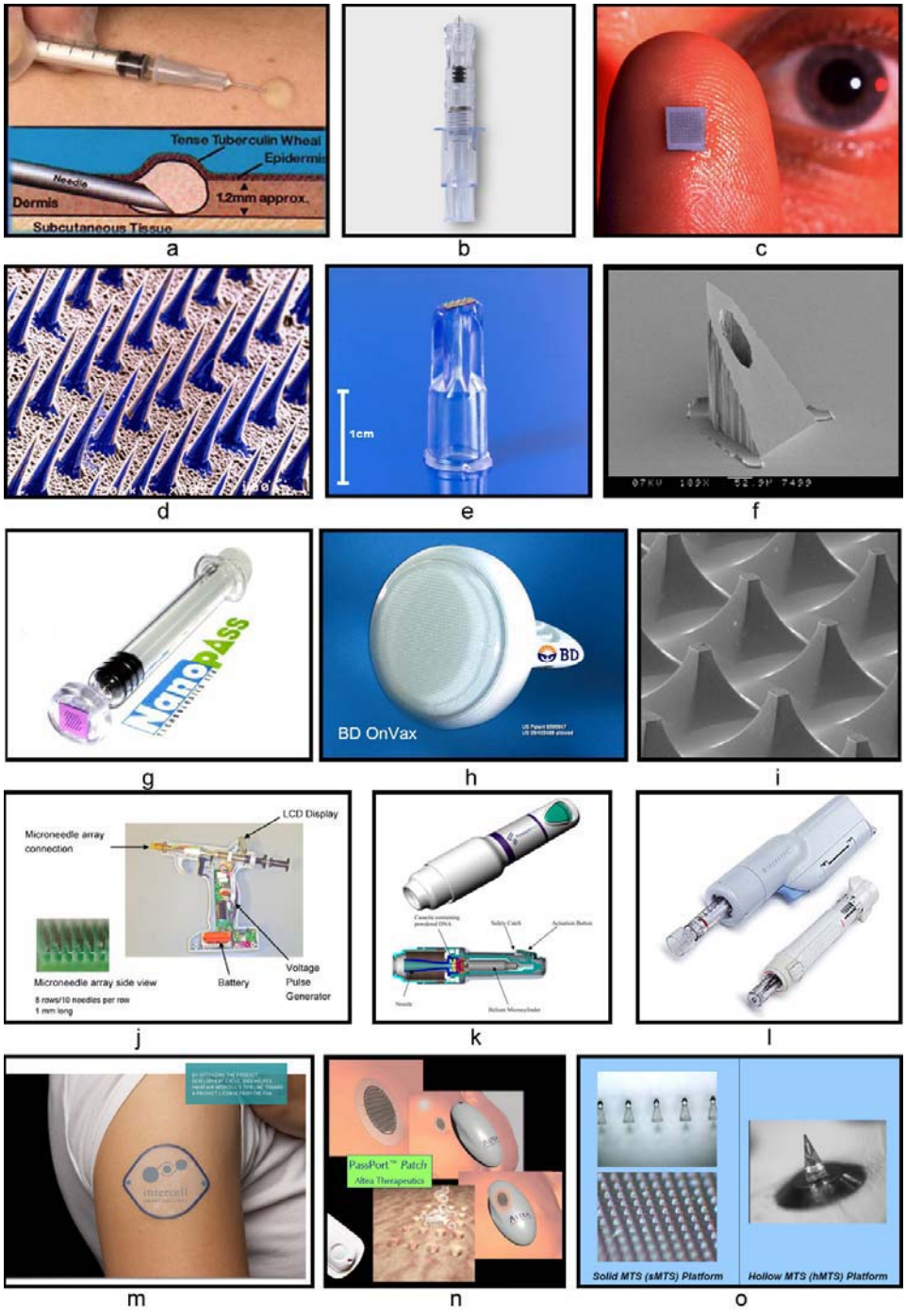

Figure 4. Approaches and devices for TCI. (a) i.d. immunization; (b) Soluvia™ (BD) [103]; (c & d) Micro-Trans™ , solid microneedle array and its SEM image [104]; (e, f & g) hollow microneedle array, MicroJet® (NanoPass) [105, 106]; (h & i) blunt-tipped microneedle array, OnVax® (BD) and its EM image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

4.1.1. Intradermal injection

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

4.1.2. Microneedle arrays

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

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

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

Solid microneedle arrays

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

Coated microneedle arrays

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

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

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

Hollow microneedle arrays

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

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

Dissolvable microneedle arrays

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

Combined approaches using microneedle arrays

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

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

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

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

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

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

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

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

4.1.3. Tape-stripping

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

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

4.1.4. Jet injection

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

4.1.5. Ultrasound

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

4.1.6. Electroporation

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

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

4.1.7. Thermo-ablation

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

4.1.8. Chemical approaches

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

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

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

4.1.9. Deformable vesicular antigen delivery systems

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

Transfersomes®

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

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

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

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

Other elastic vesicles

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

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

Table IV. New technologies targeting vaccine delivery into the skin

a. Microneedle related approaches

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

b. Other physical and chemical approaches

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

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

4.2. Immune potentiators and modulators

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

4.2.1. Bacterial exotoxins

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

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

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

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

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

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

4.2.2. CpG

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

4.2.3. LPS

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

4.2.4. Virus-like particles

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

4.2.5. Quil A

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

4.3. Combined approaches for improving TCI

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

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

4.4. Safety concerns

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

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

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

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

4.5. Concluding remarks

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

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