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Review

Frontiers of transcutaneous vaccination systems: Novel technologies and devices for vaccine delivery

Kazuhiko Matsuo^{a,b}, Sachiko Hirobe^a, Naoki Okada^{a,*}, Shinsaku Nakagawa^{a,*}

^a Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

^b Division of Chemotherapy, Kinki University Faculty of Pharmacy, 3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan

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ABSTRACT

Transcutaneous immunization (TCI) systems that use the skin's immune function are promising needle-free, easy-to-use, and low-invasive vaccination alternative to conventional, injectable vaccination methods. To develop effective TCI systems, it is essential to establish fundamental techniques and technologies that deliver antigenic proteins to antigen-presenting cells in the epidermis and dermis while overcoming the barrier function of the stratum corneum. In this review, we provide an outline of recent trends in the development of techniques for the delivery of antigenic proteins and of the technologies used to enhance TCI systems. We also introduce basic and clinical research involving our TCI systems that incorporate several original devices.

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Abbreviations: Ag, antigen; APC, antigen-presenting cell; CSSS, cyanoacrylate skin surface stripping; CT, cholera toxin; DC, dendritic cell; dDC, dermal dendritic cell; DT, diphtheria toxoid; ELISA, enzyme-linked immunosorbent assay; EpCAM, epithelial cell adhesion molecule; FITC, fluorescein isothiocyanate; FL, flexible liposome; HA, hemagglutinin; ICDRG, International Contact Dermatitis Research Group; IMI, intramuscular immunization; INI, intranasal immunization; LC, Langerhans cell; LT, heat-labile enterotoxin; MH, MicroHyal; OVA, ovalbumin; PAMPs, pattern-associated molecular patterns; SC, stratum corneum; SCI, subcutaneous immunization; SPS, skin preparation system; TCI, transcutaneous immunization; TR, TexasRed; TT, tetanus toxoid.

* Corresponding authors. Tel.: +81 6 6879 8176; fax: +81 6 6879 8176.

E-mail addresses: okada@phs.osaka-u.ac.jp (N. Okada), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

1. Introduction

Infectious disease is the most common cause of death, accounting for approximately one-third of fatalities worldwide. Recent waves of transnational migration of people and materials enhanced by the development of transportation facilities, changes in social structure, and war have increased the global spread of emerging infections, such as severe acute respiratory syndrome and avian influenza virus [1,2]. In addition, declining sanitation and the onset of drug-resistant pathogenic organisms have increased the spread of re-emerging infectious diseases, such as tuberculosis and malaria [3,4]. Although major treatment for these infectious diseases is antibiotic administration, the only fundamental prophylaxis is vaccination for a biological preparation that improves immunity to a particular disease. Vaccine development, which has a long history, has progressed recently with the development of new approaches and technologies based on advances made in the fields of bacteriology, virology, and molecular biology.

Conventional vaccination is, however, performed mainly by injection, which has several inherent problems: pain, the need for trained personnel, associated needle-related diseases or injuries, and storage or transport issues. In some areas, vaccine coverage against infection is low due to failure in follow-up as well as a lack of trained medical personnel and facilities. The reuse of needles causes the death of at least 1.3 million people per year from hepatitis B and AIDS [5]. Thus, the development of needle-free, easy-to-use, and low-invasive vaccination methods is an urgent task. With its advantages that overcome the inherent problems

of vaccination by injection, transcutaneous immunization (TCI) or intranasal immunization (INI) is now attracting attention as an alternative vaccination route.

INI, which is needle-free vaccination method, is highly expected as a hopeful vaccination procedure to stimulate both mucosal and systemic immune responses. The mucosal antigen (Ag)-specific immune response, however, is weak, thus it is necessary to develop a mucosal vaccine adjuvant to develop mucosal vaccines. The cholera toxin (CT) and heat-labile enterotoxin (LT) are potent mucosal adjuvants, but recent reports showed that a human vaccine containing inactivated influenza virus and LT as an adjuvant resulted in a very high incidence of Bell's palsy [6]. Therefore, mucosal vaccine adjuvants with high efficacy and safety for the purpose of a clinical application are necessary.

The skin has important immune functions as a pro-inflammatory organ [7–9]. The epidermis and dermis are highly populated by dendritic cells (DCs), which are potent Ag-presenting cells (APCs) with important immunostimulatory and migratory activities (Fig. 1). Langerhans cells (LCs) in the epidermis and dermal DCs (dDCs) in the dermis are important for the induction of Ag-specific immune responses in the TCI system. Thus, if Ag can be efficiently delivered to LCs or dDCs resident in the epidermal layer or dermis, TCI might elicit an effective immune response. However, there is a difficulty to overcome for development of TCI system. The uppermost layer of the epidermis is the stratum corneum (SC), which consists of about 20 layers of flattened, enucleate, and keratin-filled corneocytes surrounded by lamellae of around eight lipid bilayers [10,11]. The lipid bilayers consist

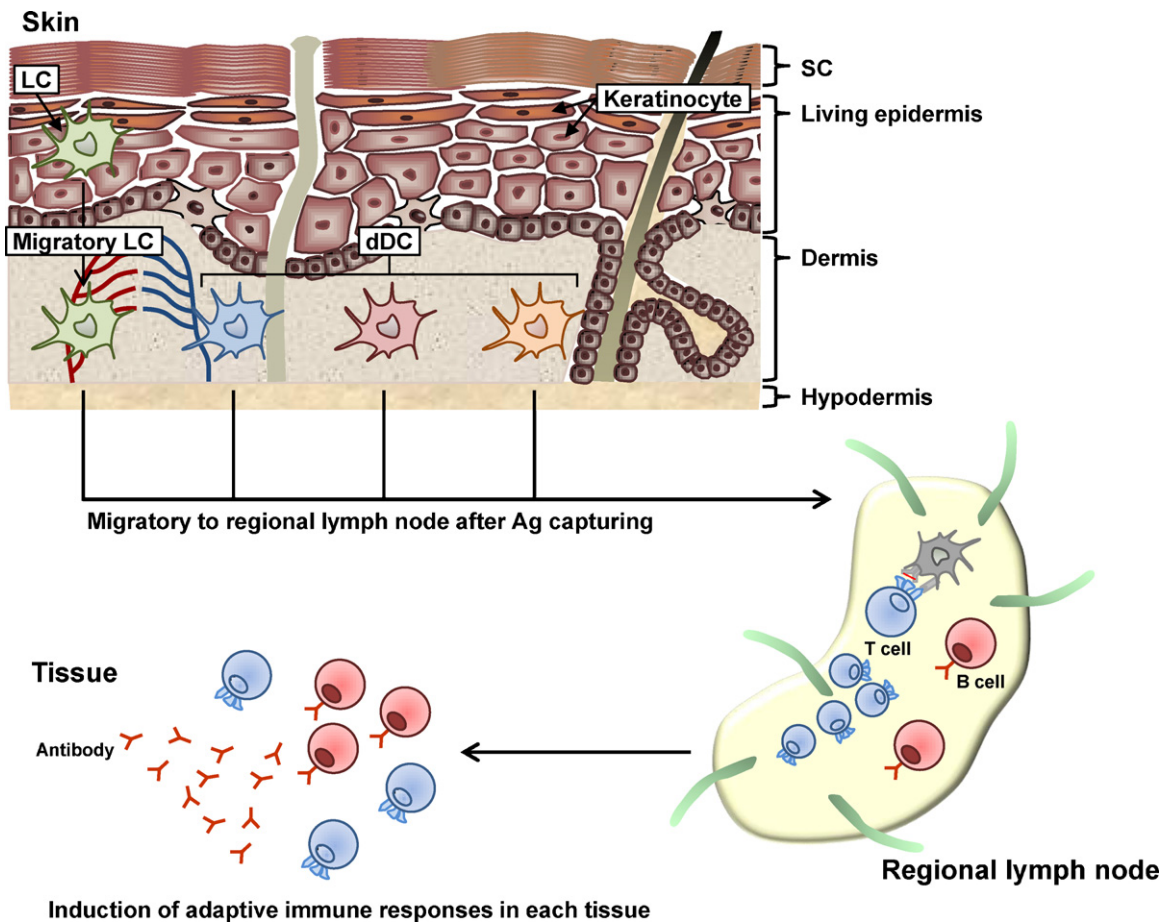


Fig. 1. Skin immune system. The skin is enriched with various immunocompetent cells such as LCs, keratinocytes, and several dDCs. Keratinocytes are mainly involved in the induction of innate immunity. LCs and dDCs capture external Ag, migrate into regional lymph nodes, present Ag to T cells, and activate Ag-specific T cells and B cells. Activated T cells and B cells migrate to each tissue and induce Ag-specific immune responses.

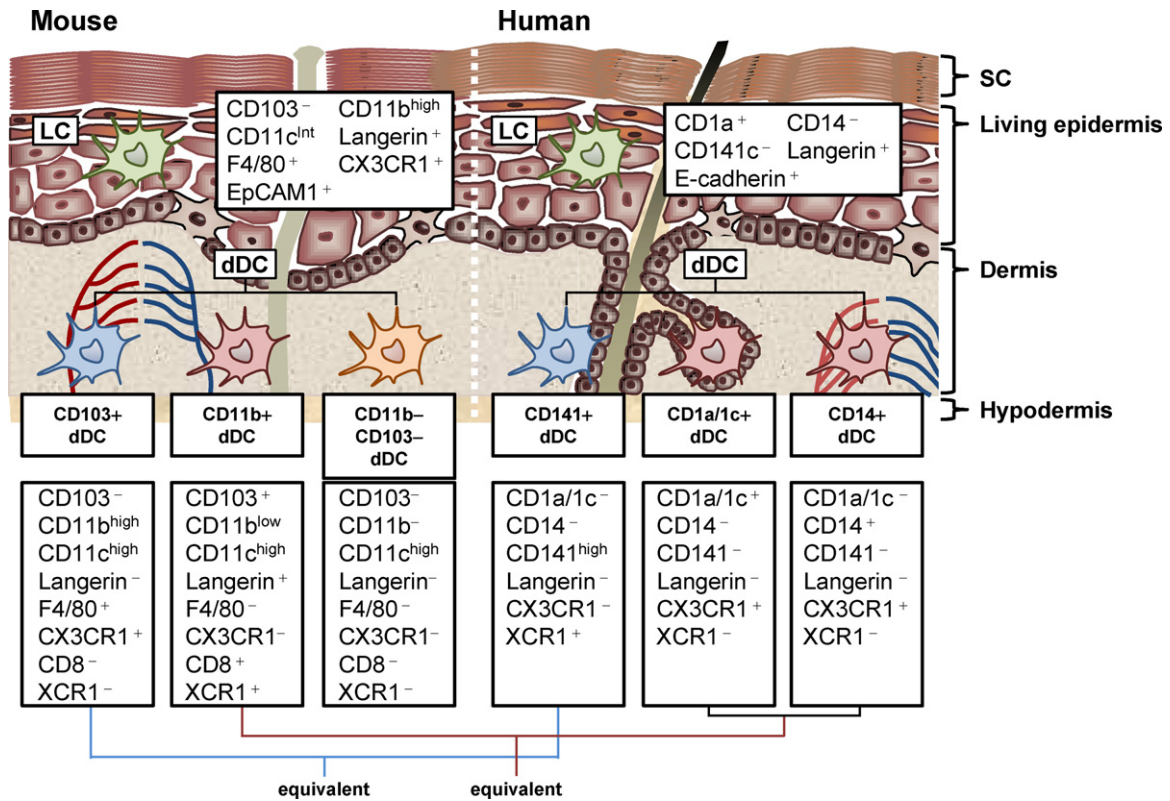


Fig. 2. Several types of skin-resident professional APCs in mice and humans. The skin contains two main populations of LCs in the epidermis and dDCs in the dermis in mice and humans. Murine dDCs and human dDCs can be distinguished into several subsets on the basis of phenotype and function; CD103-positive dDC, CD11b-positive dDC, and CD103-negative, CD11b-negative dDCs in mice, and CD1a/1c-positive dDC, CD14-positive dDCs, and CD141-positive dDCs in humans.

primarily of cholesterol, free fatty acids, and ceramides. As the SC is the principal barrier to the penetration of substances, it is difficult to efficiently deliver adequate Ag to cutaneous APCs through the SC by just applying Ag onto bare skin. Therefore, in order to develop effective TCI systems, technologies must be established that promote Ag penetration through the SC.

In this review, we outline the mechanisms of the skin immune system and recent transcutaneous antigenic protein delivery techniques, technologies, and devices. Furthermore, we introduce the progress we have made in our research into the practical application of TCI in basic, preclinical, and clinical investigations.

2. The role of the skin as an immunological organ

The skin, the access site for TCI, acts not only as a physical barrier but also as an immunologic barrier and is enriched with various immunocompetent cells such as LCs, keratinocytes, and dDCs (Fig. 1).

In special, LCs and dDCs take important roles in induction of Ag-specific immune responses. Under non-inflammatory conditions, LCs and dDCs are, for the most part, immature, meaning they have a strong endocytic capacity. When external Ag enter the skin, LCs and dDCs capture them and increase the expression of costimulatory factors, which play a role in the presentation of Ag to T cells, and CCR7 to permit the movement of APCs away from the skin and their subsequent entry into and localization within the draining lymph nodes [12]. After that, LCs and dDCs present Ag to CD4 and CD8 T cells and activate Ag-specific T cells and B cells.

Keratinocytes also involved in induction of Ag-specific immune responses by activating the innate immune system. Keratinocytes could effectively convert exogenous stimuli into host homeostatic responses [7,13]. In particular, they express numerous toll-like

receptors on their surface or in endosomes [14]. Also, another type of receptor has been discovered on keratinocytes: nucleotide binding-domain oligomerization domain-like receptors [15,16]. These receptors allow the keratinocytes to recognize bacterial components, namely, pattern-associated molecular patterns (PAMPs). In case external Ag do enter, keratinocytes produce cytokines and chemokines. TNF- α and IL-1 β constitute the signals necessary for LCs or dDCs to migrate to a regional lymph node [17]. Like keratinocytes, LCs and dDCs express these receptors that contribute to the maintenance of an inflammatory environment [18,19]. This inflammatory microenvironment, innate immunity, arises from the first contact with a vaccine component and contributes to different extents to the production of pro-inflammatory molecules that strongly contribute to the primary events of the adaptive immune response, that is, activation of skin-resident APCs. When vaccine Ag is administered into skin, Ag-specific immune responses are induced by these mechanisms. Thus, the skin is clearly an attractive organ for Ag delivery to elicit immune responses.

Several types of professional APCs inhabit the healthy skin and the studies about function of skin-resident APCs involved in induction of skin immunity have been investigated (Fig. 2). In mice, skin-resident APCs were classified into two categories; LCs in the epidermis and dDCs in the dermis. LCs and dDCs seemed to induce Th2-type and Th1-type immune responses, respectively [20,21]. However, some studies suggested that LCs were not involved in induction of immune responses [22]. In a few years, also, reports suggesting the existence of several dDC subsets have been published in mice [23,24]. It was generally assumed that the expression of langerin in the skin was strictly confined to LCs in the epidermis, but this view has been altered by current data indicating that a large population of langerin-positive cells corresponds to dDCs [25–27]. Classical langerin-negative dDCs express the macrophage

Table 1
Transcutaneous vaccine delivery techniques.

Technique	Principle	Characteristics	Ref.
Electroporation	Method to transiently increase permeability of a membrane by applying a single or multiple short-duration pulses	Advantage Activation of immunocompetent cells Disadvantages High cost, need for power-supply device, disruption of cutaneous barrier	[35–39]
Iontophoresis	Method to enhance transport of ionic or charged molecules through a biological membrane by the passage of direct or periodic electric current through an electrolyte solution with an appropriate electrode polarity	Advantage Activation of immunocompetent cells Disadvantages High cost, need for power-supply device, disruption of cutaneous barrier	[40–43]
Sonophoresis	Method to enhance substance penetration through the SC by disrupting the structure of the membrane with low-frequency ultrasound	Advantage Activation of immunocompetent cells Disadvantages High cost, need for power-supply device, disruption of cutaneous barrier	[44–47]
Jet injectors	Devices that use pressure to deliver substances into the skin	Advantage Activation of immunocompetent cells, hand-size device Disadvantage Pain, need of cold-chain	[48–52]
Patch formulations	Devices to enhance penetration of antigens into the skin	Advantage Painless, easy-to-use (self-administration) Disadvantages Poor antigen permeability, need for disruption of the SC in some cases	[53–62]
Microneedles	Devices that can create a transport pathway large enough for proteins and nanoparticles but small enough to avoid pain	Advantage Painless, easy-to-use (self-administration) Disadvantage Difficulty of fabrication, disruption of cutaneous barrier	[68–78]
Nanoparticles	Nano-bio interaction, Consequent induction of transient and reversible opening of SC, through hair follicles	Advantage Ags protected from external environment Disadvantage Low efficiency	[65,79–83]
Lipid-based vesicles	Nano-bio interaction, flexible bilayer mixes with SC and disrupts it	Advantage Ags protected from external environment Disadvantage Low efficiency	[84–88]

markers CD11b, F4/80, and CX3CR1, whereas langerin-positive dDCs express CD103, CD8 α , and XCR1 without CD11b, F4/80, or CX3CR1 [28]. It was also reported that CD103-negative, CD11b-negative dDCs exist in the dermis [29]. Especially, CD103-positive dDCs but neither dermal CD103-negative dDCs nor LCs were shown to have a crucial role in the induction of Ag-specific CD8-positive T cells (Th1-type immune responses) [30].

Recent progress was made in identifying potential homologs of mouse dDC subsets by examining human dDCs. Human skin APCs also divided into two groups on the basis of localization, LCs in the epidermis and dDCs in the dermis. Epidermal LCs preferentially induced the differentiation of CD4-positive T cells secreting Th2 cell cytokines and were efficient at crosspriming naive CD8-positive T cells [31]. Human dDCs can be distinguished into several subsets; CD1a/1c-positive dDC, CD14-positive dDCs, and CD1a/1c-negative, CD14-negative, CD141-positive dDCs by phenotype and function in the homeostatic and inflamed skin [31–34]. Human CD1a/1c-positived dDCs and CD14-positive dDCs do not express langerin and can be classified based on their reciprocal expression of CD1a and CD14, which are thought to be equivalent to mouse CD11b-positive dDCs [32]. However, the relative contributions of these subsets to the generation of immunity or tolerance are still unclear [32,33]. Yet, specialization of these different populations has become apparent. Human CD14-positive dDCs can promote antibody production by B cells [31]. In addition, CD1a/1c-negative, CD14-negative, CD141-positive DCs exhibit specialized cross-presenting function and express a number of markers associated with mouse CD103+ DCs [34].

Although the immune mechanisms of the skin remain to be completely elucidated and further analyses were should be investigated, improved knowledge of the skin immune system could

lead to the induction of optimal immune responses, such humoral immunity or cellular immunity, against infectious diseases.

3. Transcutaneous antigenic protein delivery techniques, technologies, and devices

As previously noted, the SC acts as a physical barrier against the penetration of substances into the skin. Various pharmaceutical approaches and devices have been developed to enable TCI systems to overcome the penetration barrier of the SC. In this section, the techniques, technologies, and devices used for the enhancement of TCI are reviewed (Table 1).

3.1. Electroporation

Electroporation is a method to increase the permeability of the skin by applying single or multiple short-duration pulses. It has been widely used to loosen the cell surface, allowing the delivery of molecules into living cells. With high-voltage pulses (75–100 V) delivered against the skin surface, microchannels or local transport regions are created through lipid bilayer membranes including the SC [35–39]. Zhao et al. reported that TCI with the SL8 peptide derived from ovalbumin (OVA) and CpG oligodeoxy nucleotide as an adjuvant using electroporation could induce OVA-specific T cell responses equivalent to those induced by intradermal injection [37], indicating that TCI using electroporation induced Ag-specific immune responses. However, this method requires power-supply equipment, thus they may be useful procedures in medical institutions but they cannot achieve an optimal ease of self-administration. In addition, disrupting SC as skin barriers may lead to secondary infection.

3.2. Iontophoresis

Iontophoresis is a method to enhance the transportation of ionic or charged molecules through a biological membrane by passing direct or periodic electric current through an electrolyte solution with an appropriate electrode polarity. This technique has been applied in the fields of transdermal drug delivery and has been shown by several groups to promote penetration of peptides or proteins such as insulin, calcitonin, or botulinum toxin through the SC [40,41,42]. The combination of electroporation and iontophoresis makes substance penetration even more effective [43]. From these reports, iontophoresis enhanced penetration of macro molecules through SC into skin, thus application of this method to TCI systems is expected. However, several problems about lack of convenience and risk of secondary infections remain because it requires power-supply equipment and may break cutaneous barrier.

3.3. Sonophoresis (low-frequency ultrasound)

Sonophoresis is a method to enhance substance penetration by disrupting the structure of the SC with low-frequency ultrasound. Cavitation is the formation of gaseous cavities in an ultrasound-coupling medium upon exposure to ultrasound and involves the rapid growth and collapse of a bubble (transient cavitation) or slow oscillatory motion of a bubble (stable cavitation) in the ultrasound field. Oscillations and collapse of cavitation bubbles disorder the lipid bilayers of the SC, thereby enhancing transport [44]. Dahlan et al. have shown that TCI using low-frequency ultrasound with tetanus toxoid (TT) induced anti-TT IgG and neutralizing antibodies [45,46]. Interestingly, Tezel et al. reported that ultrasound treatment induced LC activation and enhanced the Ag-specific immune response, suggesting it acts as a physical adjuvant [47]. Although TCI using sonophoresis induced Ag-specific IgG antibody and have advantage of activating immunocompetent cells, this method require power-supply equipment and disrupt cutaneous barrier, thus they have several issues in terms of usefulness and safety.

3.4. Jet injectors

Jet injectors are devices that use pressure to deliver substances into the skin [48–51]. The first devices were multiple-use nozzle jet injectors, with which a large number of patient were vaccinated through the same fluid stream and nozzle [48,49]. However, such devices are no longer used because of cross-contamination. Recent development efforts have resulted in disposable syringe jet injectors. Simon et al. reported a clinical study of the immunogenicity of trivalent inactivated influenza vaccine administered by the LectraJet M3® RA disposable syringe jet injector, which was cleared for sale and use by the U.S. Food and Drug Administration in 2009 [52]. In jet injector systems, Ag-specific immune responses are induced and administration methods are simple, but ampoules are needed in the same way as in conventional injection systems, indicating the need of a cold-chain for transport and storage.

3.5. Patch formulations

Patch formulations are one of the commonly used systems for TCI. Several groups have reported that TCI using gauze patches or adherent patches induced Ag-specific immune responses [53–57]. Application of a LT-containing single-ply polyester-rayon gauze patch onto human skin increased the anti-LT IgG titer in serum [53]. Although the other groups also have reported developing TCI systems for practical use and showed their safety and efficacy [54–57], these systems comprised a gauze patch as the TCI device. Because they require the gauze patch to be saturated with

Ag solution just before application to the skin, such TCI systems are inconvenient and require cold storage and transportation of the Ag solution, as do conventional injectable vaccination systems. In addition, the disadvantage of patch-based TCI system is the requirement of skin preparation system (SPS) or cyanoacrylate skin surface stripping (CSSS) procedures to remove SC before patch application for improvement of Ag penetration into skin. These methods may carry a risk of increasing sensitivity to secondary infection by disrupting SC as a cutaneous barrier, which is a safety issue. Thus, the development of more easy-to-use and safer patch-based TCI system is desirable.

In our research group, we have developed a hydrogel patch as a TCI device, which is made of safe materials that have already been applied to humans [58–62] and TCI formulation using a hydrogel patch was shown to induce effective immune responses to tetanus and diphtheria after application in absent of any treatment in animal models [59]. We also demonstrated its safety and efficacy by performing a clinical study of our TCI formulation for vaccination against tetanus and diphtheria in humans without disrupting SC [62].

3.5.1. Ag delivery using our hydrogel patch formulations as the TCI device

In our patch-based TCI system, we can prepare a TCI formulation by dropping Ag solution to a hydrogel patch and leaving out at room temperature for a while. The hydrogel patch formulation immersed with TexasRed (TR)-labeled OVA solution formed a concentrated Ag layer on its surface (Fig. 3A), because only water in Ag solution absorbed by hydrogel polymer.

It is very important to deliver antigenic proteins to the skin-resident APCs for induction of Ag-specific immune responses. Therefore, we analyzed biodistribution of Ag after transcutaneous administration by a hydrogel patch. There was marked penetration of the Ag into the epidermal layer of intact skin after 6-h application of a hydrogel patch containing TR-OVA to the auricle skin of mice (Fig. 3B). In human and tissue-engineered skin models, a hydrogel patch also promoted the penetration of antigenic proteins through the SC [60]. Although theories of conventional transdermal drug delivery suggest that skin structure and composition do not allow for the penetration of materials larger than 500 Da [10,11], our transcutaneous vaccination system delivered antigenic proteins (45–150 kDa) into the epidermal layer [58,59]. We proposed the following mechanisms for penetration of Ag into the skin. First, the concentrated antigenic proteins on the surface of the patch might generate a high concentration gradient of antigenic proteins in the skin, which is critical for producing the driving force needed to accelerate passive diffusion and distribution. This theory is supported by our observation that the distribution of TR-OVA in the epidermal layer was not simply a result of spreading the TR-OVA solution on the intact skin surface, and that the application of the filter paper immersed in Ag solution did not enhance either Ag penetration or antibody titer [58]. Second, humectation and hydration of the skin to which the hydrogel patch is applied might loosen intercellular gaps in the SC, which contributes to improve the penetration of water-soluble substances. According to our observations, Ag penetration via our patch system occurred mainly through the intercellular gaps of the SC. In fact, there are several reports that an increased water content in the SC leads to increased membrane fluidity and decreased electrical resistance [63,64]. Although it is possible that antigenic proteins penetrate into the epidermal layer through hair follicles – there are some reports that hair follicles allow for even nanoparticles to reach the epidermal layer in skin [65–67] – our hydrogel patch enhanced Ag penetration on a tissue-engineered skin without pores [60], suggesting that this pathway contributes little to the penetration of Ag into the skin promoted by a hydrogel patch. Through a combination of these mechanisms, our

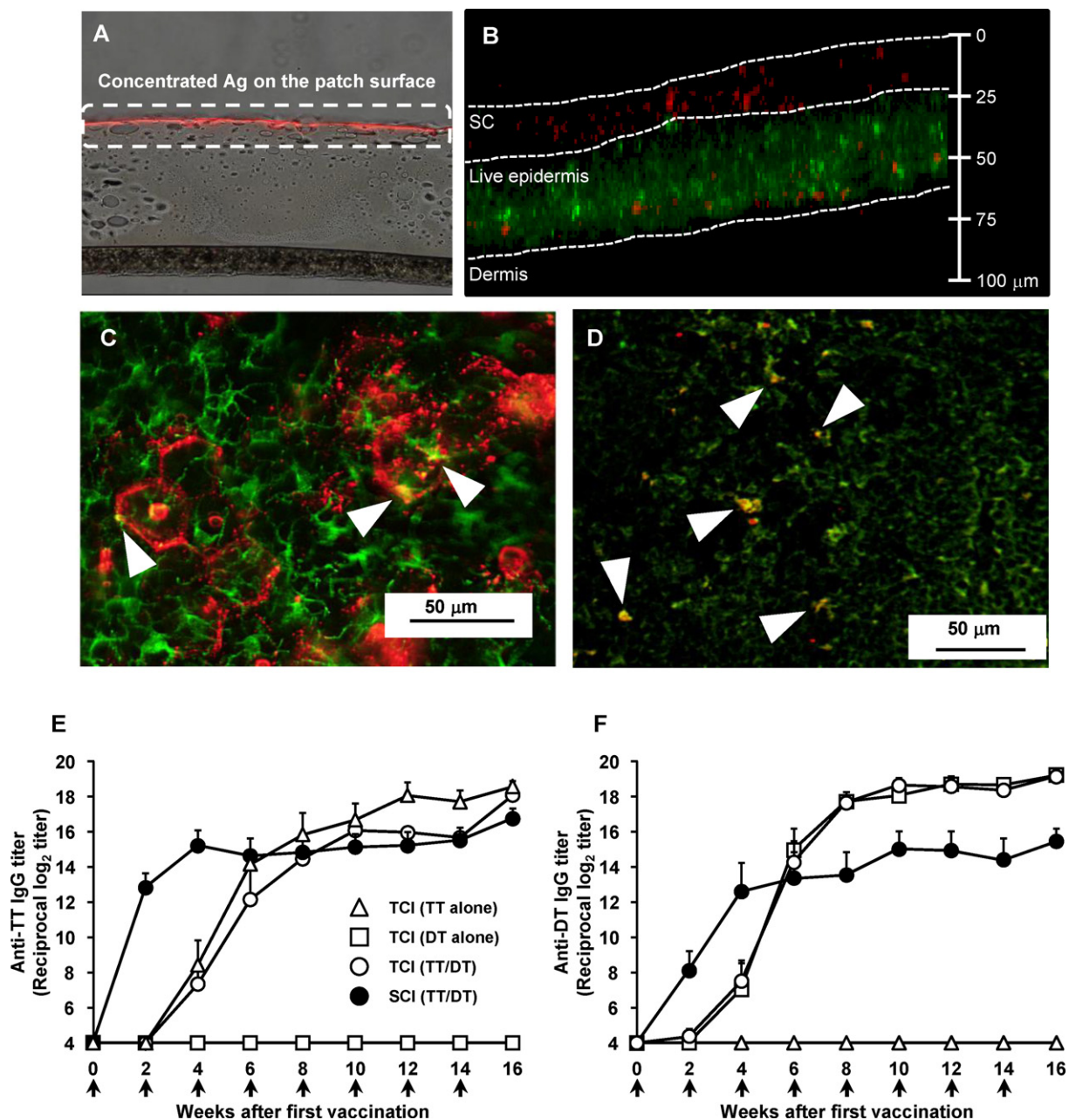


Fig. 3. Characteristics of a hydrogel patch as a TCI device. (A) Section of TR-OVA (red)-immersed hydrogel patch. (B, C, D) Localization of TR-OVA in a skin section, an epidermal sheet, and a lymph node section. TR-OVA-immersed hydrogel patches were put on auricles of C57BL/6 mice. (B) Six hours later auricles were harvested and epidermal sheets were prepared. (C) Two hours later auricles were harvested and an epidermal sheet was prepared. (D) Cervical lymph nodes were harvested 48 h after application of a patch for 24 h. The epidermal sheets and the frozen sections were stained with Alexa488-conjugated anti-mouse langerin. The epidermal sheets (B) were photographed under a confocal laser microscope. Continuous cross-sectional views were digitally superimposed. The photograph is a longitudinal section of three-dimensional images of epidermal sheets. Epidermal sheets (C) and lymph node sections (D) were photographed under a fluorescence microscope. (B–D) red: TR-OVA as Ag, green: LCs, yellow (arrowhead): merged with TR-OVA (red) and LCs (green). (E and F) TT- or DT-specific immune responses in HWY hairless rats after transcutaneous vaccination. Hairless rats were transcutaneously vaccinated with TT alone (100 μg), DT alone (100 μg), or combined TT and DT (100 μg each) for 24 h eight times at 2-week intervals. A control group was subcutaneously immunized with combined TT and DT (100 μg each) eight times at 2-week intervals. At the indicated points, serum collected from these hairless rats was assayed for IgG titers against (E) TT or (F) DT by ELISA. Data are expressed as mean ± SE of results from 10 rats. Arrows indicate vaccination points. Arrowheads indicate yellow fluorescent spots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

patch vaccine system promoted the penetration of water-soluble macromolecular proteins into the SC.

As shown in Fig. 3C and D, yellow fluorescent spots, indicating that TR-OVA localization accorded with LC localization, were observed in merged images of an epidermal sheet and lymph node sections prepared from mice with intact skin, suggesting that LCs, which are cells critical for the induction of potent immune responses, captured antigenic proteins penetrated into the skin and migrated into the regional lymph node. Thus, Ag-capturing LCs,

which migrated from the epidermal layer to regional lymph nodes, would greatly contribute to triggering and amplifying Ag-specific immune responses induced by transcutaneous vaccination using the hydrogel patch formulation.

3.5.2. Vaccination efficacy and safety of TCI using a hydrogel patch

In an animal model of tetanus and diphtheria infection, the vaccination efficacy of TCI using a hydrogel patch was evaluated. TCI

using a hydrogel patch elicited toxoid-specific immune responses and the serum titer of antibody in the TCI groups were equivalent to or greater than those of the subcutaneous immunization (SCI) group (Fig. 3E and F). In rats vaccinated with combined TT and DT, both TT and DT-specific IgG antibodies were detected in serum as efficiently as that in rats vaccinated with each toxoid alone, suggesting that our TCI using a hydrogel patch is applicable to a combination vaccine. As mixed inoculation is now recommended in vaccination, our TCI formulation is suitable for practical use. We also demonstrated that TCI using a hydrogel patch containing TT and DT induced little adverse reactions in local and systemic toxicity assessments [61], indicating that hydrogel patch-based TCI formulation is a non-invasive vaccination method. In addition, on the basis of IgG subclass analysis, it was suggested that our TCI using the hydrogel patch formulation predominantly elicited a Th2-type immune response rather than a Th1-type immune response [58,59]. Further analyses are necessary to elucidate the Th2-dominant mechanism in our patch vaccination.

In our hydrogel patch-based TCI system, we can simply prepare a manageable TCI formulation like general fomentations with a concentrated Ag layer on the surface of the hydrogel patch. Our TCI system using a hydrogel patch enhanced Ag penetration into the skin and induced Ag-specific immune responses by single application onto skin surface without disrupting SC. This is superior to other patch-based TCI formulation in terms of avoiding secondary infections by breaking skin barriers.

3.6. Microneedles

Patch formulations, such as the hydrogel patch, are less effective at promoting penetration of particulates and insoluble Ags through the SC. Most practical vaccine Ags are in a particulate state, for example, the less virulent strains of bacteria. The development of a different TCI system that is effective for use with all Ag forms is needed. A microneedle array contains many micrometer-sized needles that can create a transport pathway large enough for proteins and nanoparticles, but small enough to avoid pain [68–74]. Microneedle arrays can penetrate the SC barrier and deliver Ag to immunocompetent cells in the skin more efficiently than other TCI

systems. In addition, the use of a disposable array is suitable for self-administration. Thus, microneedles are the most attractive devices for the development of effective TCI systems. Microneedles were first conceptualized for drug delivery in a 1976 patent [75]. Since then, several type of microneedles have been developed and they are classified into four types with respect to mechanism of action: (1) solid microneedles for pretreatment of the skin to increase permeability, (2) microneedles coated with drug that dissolves in the skin, (3) polymer microneedles encapsulating the drug that fully dissolves in the skin, and (4) hollow microneedles for infusing the drug into the skin.

Traditional microneedle arrays made from silicon, metal, stainless steel, or titanium were reported in the early stages of development, but the clinical use of microneedle arrays has faced serious obstacles because needles on microneedle arrays can fracture and remain in the skin, creating a safety issue. These conventional microneedle arrays suffer from the risk of fracture of microneedle fragments in the skin, therefore, in 2004, microneedle systems made with biocompatible or biodegradable polymers began to be developed [69], and their superior safety has led to early clinical use. This system, however, remains the risk of breaking cutaneous barrier by insertion of microneedles into skin. In manufacture of dissolving microneedles, the technical innovation is required to allow Ag to be incorporated into the matrix of microneedle material using mild procedures that do not cause the decrease of antigenicity or compromise material strength.

Our research group has developed a dissolving microneedle array (MicroHyla[®]; MH) as a TCI device, which was fabricated using micromolding technologies with biocompatible sodium hyaluronate as the base material and this approach demonstrated effective vaccination effects comparable to those of conventional injection systems [76–78].

3.6.1. Characteristics of our MH as a TCI device

We have developed a dissolving microneedle array, MH as mentioned above, made of sodium hyaluronate as the base material (Fig. 4A). We successfully fabricated several types of MH in various forms and lengths: konide-shaped MH (needle length 200 or 300 μm) and cone-shaped MH (needle length 300, 500, or 800 μm)

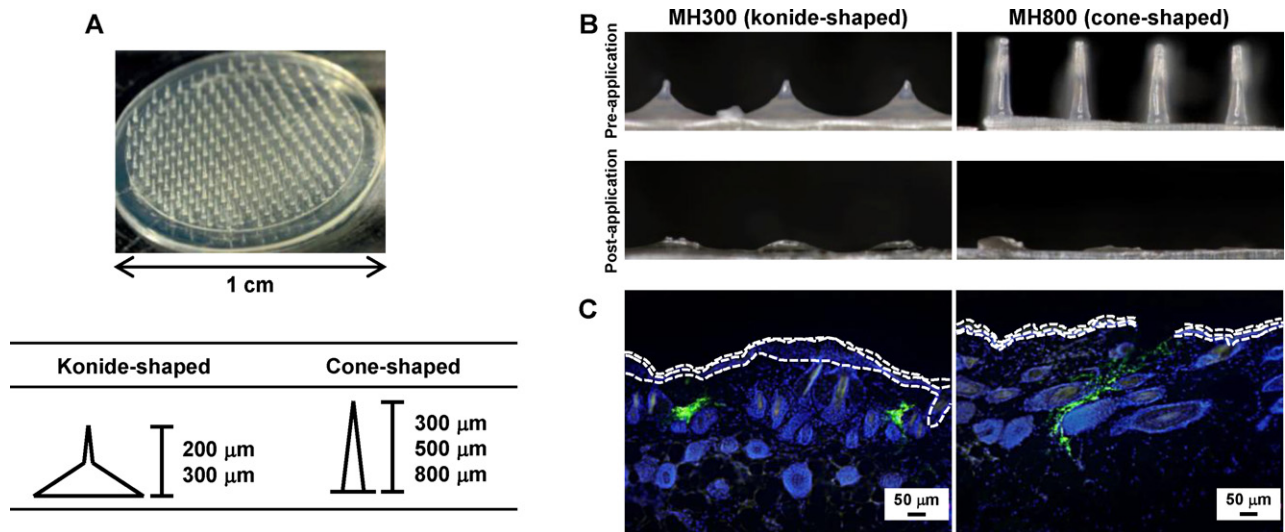


Fig. 4. Dissolving microneedle array patch (MicroHyla[®]; MH). (A) Bright-field micrograph of whole MH. There are two type of MH, konide-shaped MH (needle length: 200 or 300 μm) and cone-shaped MH (needle length: 300, 500, or 800 μm). (B) Bright-field micrograph of microneedles on konide-shaped MH300 or cone-shaped MH800 before or after insertion into skin. Each MH was applied on the back skin of BALB/c mice. One hour later, each MH was removed and photographed under a stereoscopic microscope. (C) Konide-shaped MH300 or cone-shaped MH800 encapsulating FITC-labeled silica particles were applied on the back skin of BALB/c mice and skin was harvested 6 h later. Frozen sections were photographed under a fluorescence microscope. The nucleus was counterstained with 4',6-diamidino-2-phenylindole (blue). Area between the upper dotted line and the middle dotted line is the SC, area between the middle dotted line and the lower dotted line is the living epidermis, and area below the lower dotted line is the dermis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

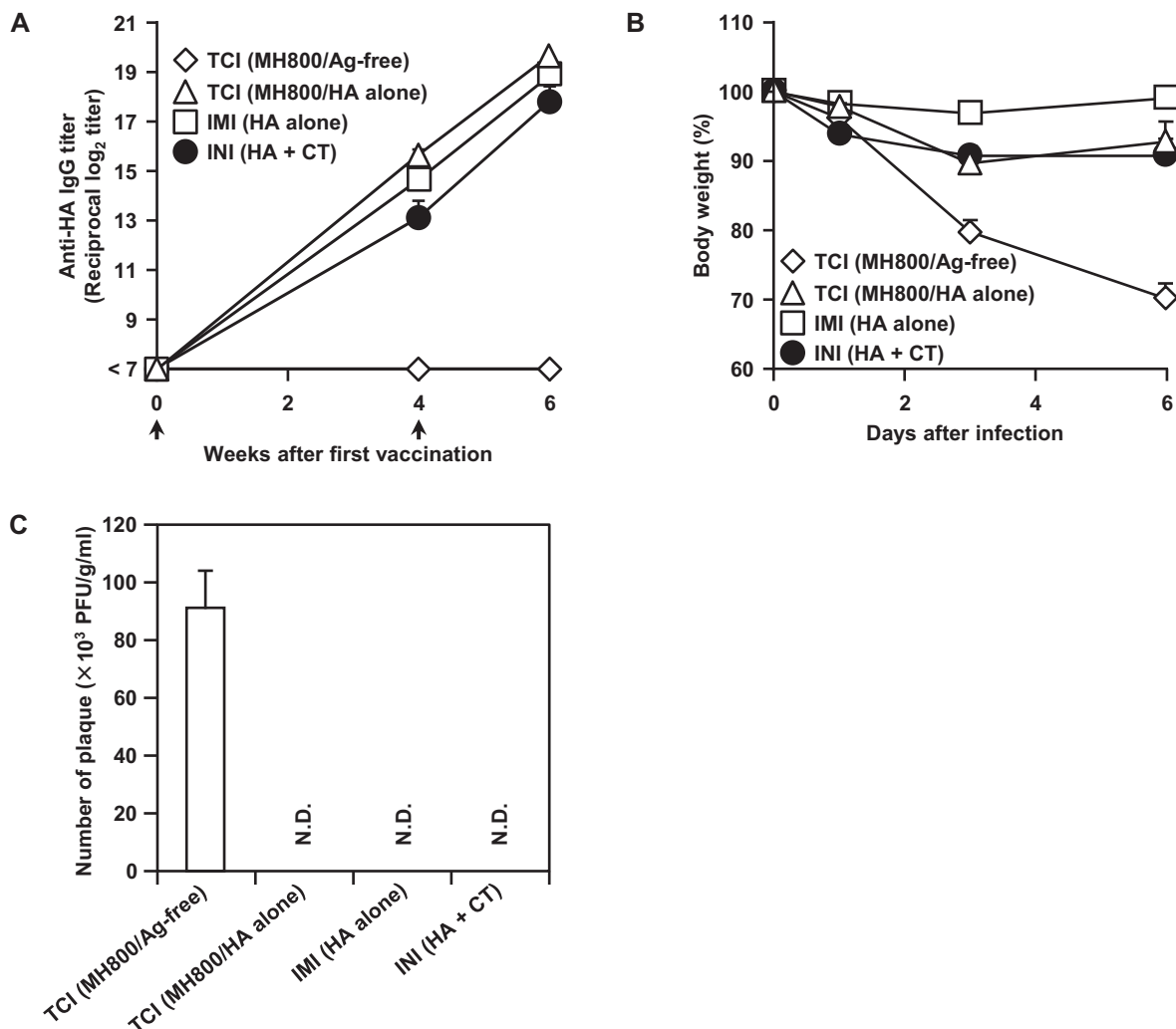


Fig. 5. Protection of vaccinated mice against challenge with influenza virus. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μ g) for 6 h twice at 4-week intervals. Control groups were treated with TCl without HA, intramuscular injection of HA (0.4 μ g), or intranasal application of HA (0.4 μ g) combined with CT (10 μ g) as an adjuvant twice at 4-week intervals. Two weeks after last vaccination, these mice were each infected intranasally with 6×10^5 PFU of the A/PR/8/34(H1N1) virus. (A) At the indicated points, sera collected from the mice were assayed for the titer of HA-specific IgG by ELISA. (B) Body weight was measured each day after infection and is presented as a percentage of the original weight before infection (day 0). (C) Six days after infection, the lungs were collected from the mice and the number of viruses in the lung homogenate was determined with a plaque assay system. Data are expressed as mean \pm SE of results from (A) 13 or (B and C) 10 mice. Arrowheads indicate vaccination points.

(Fig. 4A). The microneedles on the MH were dissolved by water in the skin and thus had no danger of remaining in the skin, making our MH safer than traditional microneedle arrays made of metal or stainless steel. In fact, the microneedle tips were fully dissolved at 1 h (Fig. 4B). Application of each MH caused only temporary skin irritation and the skin barrier function after insertion recovered immediately [76], suggesting that the holes caused by insertion of each MH closed up quickly. These results suggest low probability of causing secondary infection by application of MH. In observation of skin sections after application of each MH containing fluorescein isothiocyanate (FITC)-silica particles, they were clearly detected (Fig. 4C), suggesting that the MH delivered particulate Ag into the epidermis or dermis without regard for the Ag form. In addition, the MH size can be used to control the depth of Ag delivery, meaning that each MH might deliver Ag to specific skin-resident APCs, LCs in the epidermis or several dDCs in the dermis.

3.6.2. Vaccination efficacy and safety of TCl using MH

We examined the efficacy of vaccination with influenza hemagglutinin (HA) Ag, which is particulate Ag. In an influenza virus

challenge, TCl with HA alone elicited production of HA-specific functional IgG antibody equivalent to that after intramuscular immunization (IMI) with HA alone or INI with combined HA and CT as an adjuvant (Fig. 5A). On the other hand, little anti-HA IgA antibody was detected in the TCl and IMI groups [77]. After challenge with A/PR/8/34 influenza virus, mice in the TCl group showed no remarkable weight loss, similar to those in the IMI group and INI with CT group (Fig. 5B). In addition, the virus titer in the lungs of the TCl group was below the detection limit (Fig. 5C), demonstrating that our TCl system provided protection equal to that of IMI or INI with adjuvant. In INI system, mucosal vaccine adjuvants with high efficacy and safety for the purpose of a clinical application are necessary. As compared to INI system, our TCl could efficiently elicit Ag-specific vaccine effect without an adjuvant, which is an advantage of our TCl system.

In addition, the vaccination efficacy of TCl using MH was also demonstrated in tetanus, diphtheria, and malaria infection models. On the basis of these results, TCl system using MH suggested to induce Ag-specific immune responses against any vaccine Ags, such as soluble Ags, insoluble Ags, or particulate Ags, which conventional TCl system fail to do so.

Table 2
Clinical studies of TCI.

Device	Antigens	Dose	Phase	Results	Ref.
Patch (SPS)	LT	37.5 µg	II	<ul style="list-style-type: none"> • Safety of vaccination technique • Induction of anti-LT antibody titer • 75% protection against moderate <i>E. coli</i> diarrhea, 84% against acute <i>E. coli</i> diarrhea 	[53]
Patch (CSSS)	Live-attenuated measles	10 ³ pfu	I/II	<ul style="list-style-type: none"> • Pretreatment with tape-stripping procedures • Safety • Induction of Ag-specific salivary IgA • Detection of Ag-specific IFN-γ-producing T cells 	[54]
Patch (CSSS)	Inactivated influenza/tetanus vaccine	15 µg	I	<ul style="list-style-type: none"> • Pretreatment of abrasion by emery paper with 10% glycerol and 70% alcohol • Safety • Better seroconversion rate than IMI 	[90]
Patch (hydrogel patch)	TT and DT	2 mg each	Clinical study	<ul style="list-style-type: none"> • Safety • Induction of neutralizing IgG antibody 	[62]
Hollow microneedle	Inactivated influenza vaccine	3–6 µg	I	<ul style="list-style-type: none"> • Mild and transient local reaction • Induction of immunogenic responses 	[91]

Thus, we can conclude that our TCI system using MH which is dissolved in the skin effectively confers protective immunity without causing serious adverse reactions in an animal model.

Conventional microneedle array made of metal or stainless steel has difficulties in clinical application because needles on microneedle arrays can fracture and remain in the skin, which is serious problem. However, the microneedles on the MH were dissolved by water in the skin and thus had no danger of remaining in the skin, indicating that TCI using MH would be attractive vaccination method in terms of both safety and efficacy.

3.7. Nanoparticles

Recent studies suggested that nanoparticles are attractive means for transcutaneous Ag delivery. By disrupting the SC as a result of the nano-bio interaction with skin lipids, antigenic proteins encapsulated in the nanoparticles can be delivered through the SC into the skin. Some researchers reported that nanoparticle vaccine compounds can penetrate via the hair follicles where there is a high density of APCs and enhanced immune responses. There are numerous nanoparticle systems available, including polymeric poly (D-L-lactic-co-glycolic acid) and poly (lactic acid) nanoparticles, biodegradable chitosan nanoparticles, and metal nanoparticles [65,79–83].

3.8. Lipid-based vesicles

In addition, lipid-based vesicles such as liposomes, transferosomes, or niosomes have structures similar to those of biological membranes and facilitate skin penetration [84–88]. When mixed with SC lipids, flexible liposomes (FLs) can carry a remarkable amount of lipid mass into the skin and can, therefore, be advantageous in promoting cutaneous drug disposition after disrupting the skin barrier with their flexible bilayers [88]. It also has been reported that FLs stimulated a transcutaneous immune response by acting as an adjuvant [89].

The design of novel formulations especially nanoscale systems, such as nanoparticles and lipid-based vesicles, can be helpful for protecting the Ag from external environment and keeping the long term activity. These properties are conducive to the application of transcutaneous vaccine. However, the development of novel nanoscale systems for TCI is limited by the low efficiency in eliciting robust immune responses.

4. Clinical studies of transcutaneous vaccination

For the diffusion of the vaccine worldwide including in developing countries, patch formulations and microneedles are more suitable because of their ease of use and efficacy. Several research groups have conducted clinical studies of TCI using patch formulations or microneedle systems in recent years (Table 2). Glenn et al. first reported the results of TCI using a patch in humans [55]. Application of a patch containing LT as Ag resulted in robust LT-specific antibody responses. In addition, their group used LT to investigate patch vaccination against traveler's diarrhea in a phase II clinical trial and found that the 59 LT-patch recipients were protected against moderate-to-severe diarrhea (protective efficacy [PE] 75%) and severe diarrhea (PE 84%) [53]. LT-patch recipients who became ill had shorter episodes of diarrhea (0.5 vs 2.1 days) with fewer loose stools (3.7 vs 10.5) than recipients of placebo [55]. Since then, numerous studies of devices that serve as simple, easy-to-use, and low-invasive TCI systems have been undertaken. Etchart et al. showed that TCI of human adult volunteers with live-attenuated measles induced Ag-specific immune responses in their phase I/II clinical study [54]. Combadiere et al. demonstrated that TCI with an inactivated influenza vaccine induced a significant increase in influenza vaccine-specific CD8 responses compared with those obtained from the intramuscular route [90]. However, these TCI systems require cyanoacrylate skin surface stripping for Ag delivery into skin, which might cause skin irritation as one of the side effects.

Microneedle-based TCI systems have also been applied in clinical trials. Van Damme et al. reported the results of a clinical study of influenza vaccination in which a hollow microneedle device (MicronJet) was used [91]. Local adverse reactions were significantly more frequent than those with intramuscular vaccination, but were mild and transient in nature. After TCI, immunogenic responses increased in humans. In addition, the safety and efficacy of several microneedle devices have been assessed in applications other than vaccination [92,93]. In the future, more clinical studies will be conducted for needle-free, easy-to-use, low-invasive, and low-cost vaccination methods.

We performed a clinical study of our original hydrogel patch formulation containing combined TT and DT in humans (Fig. 6A and B) [62]. In the safety assessment to evaluate local adverse responses at 0 h and 24 h after patch removal, a TCI formulation containing TT and DT was shown not to induce local severe adverse events (Fig. 6C). As shown in Fig. 6D, anti-TT IgG and anti-DT IgG increased (paired-*t* test; *p* < 0.01) following the first vaccination using the

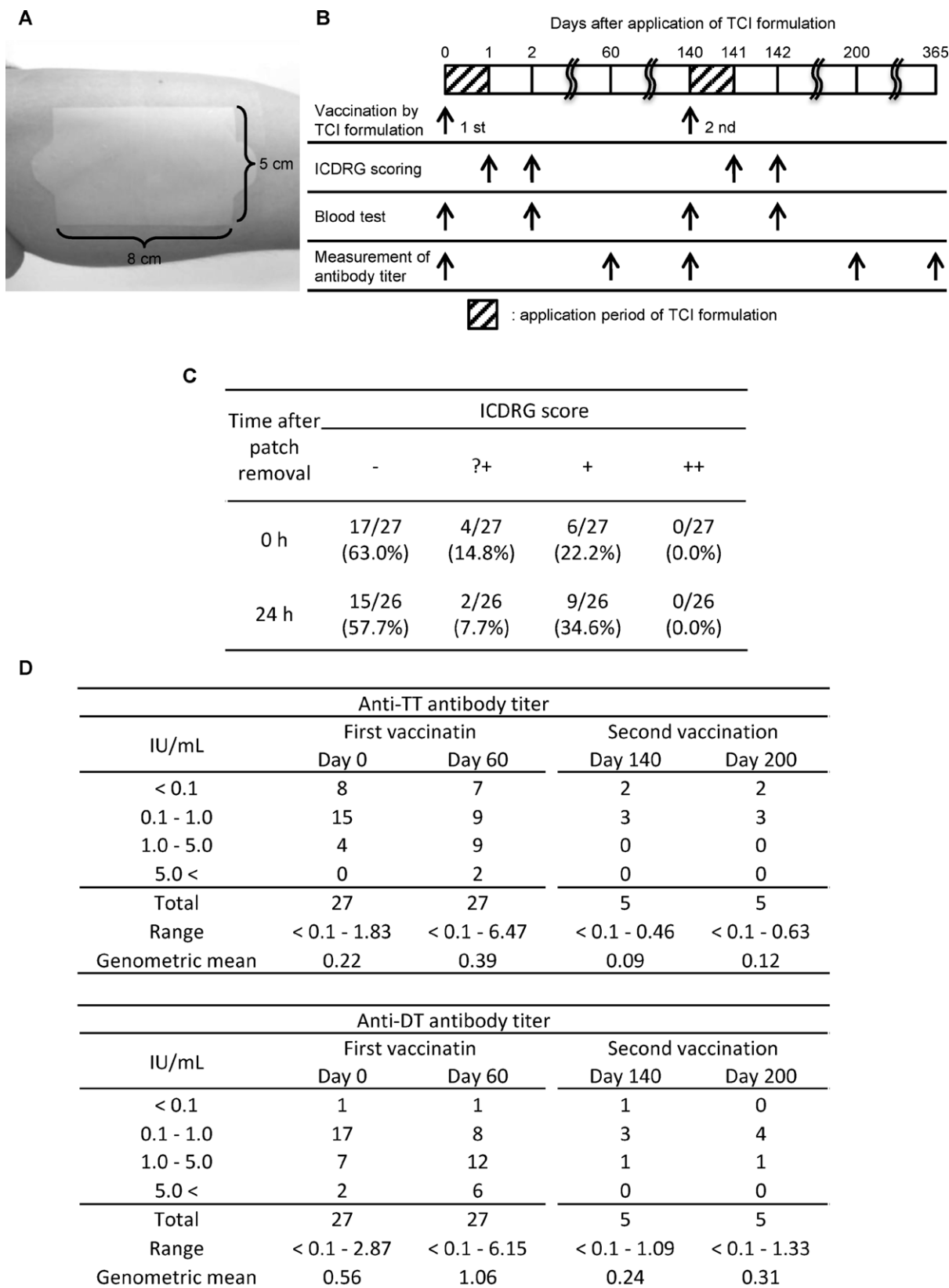


Fig. 6. Clinical study of a TCI formulation using a hydrogel patch. (A) A hydrogel patch (5 cm × 8 cm) containing TT and DT (2 mg each) was applied on the left brachial medial skin for 24 h. (B) Experimental design about clinical study of TCI formulation using a hydrogel patch containing TT and DT. Each experiment was conducted at the indicated points. (C) Local adverse events after applying the TCI formulation. Twenty-four hours after application, the TCI formulation was removed from the investigational sites. Skin irritation reactions were scored according to the classification of the International Contact Dermatitis Research Group (ICDRG) to assess local adverse responses at 0 h and 24 h after removal. The data represent the number and percent of subjects who showed each symptom. -: negative reaction; ?+: doubtful reaction (faint erythema only); +: weakly (non-vesicular) positive reaction (erythema, infiltration, and possibly papules); ++: strongly (vesicular) positive reaction (erythema, infiltration, papules, and vesicles). (D) Toxoid-specific IgG titer before (Day 0 or Day 140) and 60 days (Day 60 or Day 200) after first or second application of TCI formulation. At indicated points, serum was collected and anti-TT or DT IgG titer was determined by ELISA.

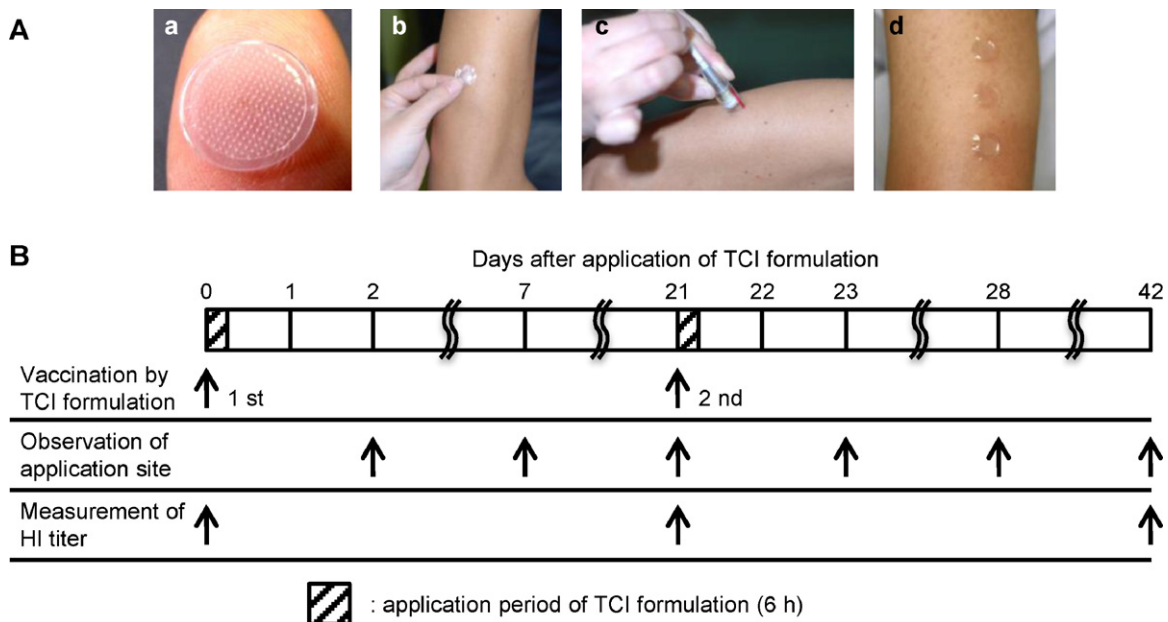


Fig. 7. Clinical study of a TCI formulation using a MH. (A) Schematic drawing of transcutaneous vaccination procedure. a: bright-field micrograph of whole MH, b: putting MH on left brachial lateral skin. c: Application of MH with an applicator. d: Covering with an adhesive tape. (B) Experimental design about clinical study of TCI formulation using a MH containing trivalent influenza HA Ags. Each experiment was conducted at the indicated points.

TCI formulation, indicating that a single application of our TCI formulation could induce an immune response in humans. We also administered a second vaccination to five subjects in whom neither antibody titer was significantly increased by the first vaccination. The IgG titers increased in a part of subjects following the second vaccination, suggesting that an additional application increases the efficacy of the TCI formulation. Antibody titers on day 365 after application of the TCI formulation were maintained at a higher level than those on day 0 in all subjects examined, although antibody titers tended to be lower on day 365 than on day 60 [62]. Conventional patch-based TCI systems require the pre-treatment of disrupting or removing SC, but our hydrogel patch achieved Ag penetration into skin without removing the SC and Ag-specific antibodies were produced in some subjects by a single application in humans, which represents a safety and efficacy advantage.

We also conducted a clinical evaluation of TCI using MH (Fig. 7). Ag-free konide-shaped MH300, cone-shaped MH500, and cone-shaped MH800 as TCI devices were applied on left brachial lateral skin (Fig. 7A) and they caused no serious local or systemic adverse reactions (in preparation). To evaluate the efficacy of vaccination (Fig. 7B), we used trivalent influenza HA Ags. HA-containing cone-shaped MH800 induced HA-specific IgG responses against three HA Ags without severe adverse events (in preparation), indicating that our MH-based TCI system was safe and efficacious in humans.

These simple, easy-to-use, low-invasive, and effective TCI formulations might be applicable for mass treatment in the event of an outbreak and for increasing vaccination rates in developing countries. We expect that our TCI system as an innovative vaccination method will be put to practical use at an early date and greatly will contribute to decrease the mortality and morbidity by infectious diseases.

5. Conclusion and future perspectives

The development of vaccines, which represent the only basic prophylaxis against infectious diseases, is drawing attention worldwide. The main objective of vaccine development is the establishment of manufacturing technologies that supply safe and effective vaccine Ag rapidly and stably, but the problem of how

to carry out enough vaccinations to prevent infectious diseases remains to be solved. In order to distribute the vaccine across the world to people who need it, especially those in developing countries, easy-to-use, low-cost, and low-invasive vaccination methods instead of conventional injection systems are required. TCI offers an attractive avenue for the development of needle-free prophylaxis. The main challenge to be addressed during the development of TCI systems is to ensure accurate delivery of Ag to the epidermis and dermis through the SC. As we introduced in this review, various approaches to overcome the SC barrier have been developed and basic, preclinical, or clinical studies of these approaches have been conducted.

Recent studies have demonstrated that intradermal vaccine delivery to skin-resident APCs can increase the magnitude of the immune response rather than IMI. For example, some studies evaluating intradermal delivery of influenza vaccine have suggested that dose sparing relative to IMI can be achieved [94,95]. Nowadays, INTANZA®/IDFlu® is marketed as a new trivalent inactivated influenza vaccine administered by the intradermal route. Thus, TCI systems targeting the skin immune system are attractive vaccination methods that can supplant conventional IMI or SCI in terms of not only ease and safety but also efficacy.

Practical use of these easy-to-use, low-cost, low-invasive, and effective transcutaneous vaccination methods in the near future would contribute to a global countermeasure against infectious disease and would greatly benefit countries with poor vaccination rates.

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References

- Leppin A, Aro AR. Risk perceptions related to SARS and avian influenza: theoretical foundations of current empirical research. *Int J Behav Med* 2009;16:7–29.
- Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). *N Engl J Med* 2005;352:333–40.
- Valadas E, Antunes F. Tuberculosis, a re-emergent disease. *Eur J Radiol* 2005;55:154–7.
- Campbell CC. Malaria: an emerging and re-emerging global plague. *FEMS Immunol Med Microbiol* 1997;18:325–31.
- Miller MA, Pisani E. The cost of unsafe injections. *Bull World Health Organ* 1999;77:808–11.
- Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med* 2004;350:896–903.
- Sugita K, Kabashima K, Atarashi K, Shimauchi T, Kobayashi M, Tokura Y. Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T cells in the skin. *Clin Exp Immunol* 2007;147:176–83.
- Mathers AR, Larregina AT. Professional antigen-presenting cells of the skin. *Immunol Res* 2006;36:127–36.
- Sen D, Forrest L, Kepler TB, Parker I, Cahalan MD. Selective and site-specific mobilization of dermal dendritic cells and Langerhans cells by Th1- and Th2-polarizing adjuvants. *Proc Natl Acad Sci U S A* 2010;107:8334–9.
- Barry BW. Breaching the skin's barrier to drugs. *Nat Biotechnol* 2004;22:165–7.
- Bos JD, Meinardi MM. The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp Dermatol* 2000;9:165–9.
- Saeki H, Moore AM, Brown MJ, Hwang ST. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol* 1999;162:2472–5.
- Wu C, Li C, Wei L, Zheng Z. Innate immune modulation of keratinocytes by anti-keratin 16 antibodies. *Exp Dermatol* 2008;17:645–52.
- Kawai K. Expression of functional toll-like receptors on cultured human epidermal keratinocytes. *J Invest Dermatol* 2003;121:217–8.
- Harder J, Nunez G. Functional expression of the intracellular pattern recognition receptor NOD1 in human keratinocytes. *J Invest Dermatol* 2009;129:1299–302.
- Kobayashi M, Yoshiki R, Sakabe J. Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes. *Br J Dermatol* 2009;160:297–304.
- Saalbach A, Klein C, Schirmer C, Briest W, Andereg U, Simon JC. Dermal fibroblasts promote the migration of dendritic cells. *J Invest Dermatol* 2010;130:444–54.
- Flacher V, Bouschbacher M, Verronè E, Massacrier C, Sisirak V, Berthier-Vergnes O, et al. Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria. *J Immunol* 2006;177:7959–67.
- Renn CN, Sanchez DJ, Ochoa MT, Legaspi AJ, Oh CK, Liu PT, et al. TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response. *J Immunol* 2006;177:298–305.
- Tada Y, Asahina A, Fujita H, Sugaya M, Tamaki K. Langerhans cells do not produce interferon- γ . *J Invest Dermatol* 2003;120:891–2.
- Fujita H, Asahina A, Sugaya M, Nakamura K, Gao P, Fujiwara H, et al. Differential production of Th1- and Th2-type chemokines by mouse Langerhans cells and splenic dendritic cells. *J Invest Dermatol* 2005;124:343–50.
- Romani N, Brunner PM, Stingl G. Changing views of the role of Langerhans cells. *J Invest Dermatol* 2012;132:872–81.
- Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol* 2008;8:935–47.
- Helft J, Ginhoux F, Bogunovic M, Merad M. Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice. *Immunol Rev* 2010;234:55–75.
- Ginhoux F, Collin MP, Bogunovic M, Abel M, Leboeuf M, Helft J, et al. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J Exp Med* 2007;204:3133–46.
- Poulin LF, Henri S, de Bovis B, Devillard E, Kissenpfennig A, Malissen B. The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. *J Exp Med* 2007;204:3119–31.
- Bursch LS, Wang L, Igyarto B, Kissenpfennig A, Malissen B, Kaplan DH, et al. Identification of a novel population of Langerin+ dendritic cells. *J Exp Med* 2007;204:3147–56.
- Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The origin and development of nonlymphoid tissue CD103+DCs. *J Exp Med* 2009;206:3115–30.
- Shklovskaya E, Roediger B, Fazekas de St Groth B. Epidermal and dermal dendritic cells display differential activation and migratory behavior while sharing the ability to stimulate CD4+T cell proliferation in vivo. *J Immunol* 2008;181:418–30.
- Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, et al. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol* 2009;10:488–95.
- Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, et al. Functional specializations of human epidermal Langerhans cells and CD14+dermal dendritic cells. *Immunity* 2008;29:497–510.
- Lenz A, Heine M, Schuler G, Romani N. Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization. *J Clin Invest* 1993;92:2587–96.
- Angel CE, Lala A, Chen CJ, Edgar SG, Ostrovsky LL, Dunbar PR. CD14+ antigen-presenting cells in human dermis are less mature than their CD1a+ counterparts. *Int Immunol* 2007;19:1271–9.
- Haniffa M, Shin A, Bigley V, McGovern N, Teo P, See P, et al. Human tissues contain CD141^{hi} cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity* 2012;37:60–73.
- Prausnitz MR, Bose VG, Langer R, Weaver JC. Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery. *Proc Natl Acad Sci U S A* 1993;90:10504–8.
- Vanbever R, Lecouturier N, Pr at V. Transdermal delivery of metoprolol by electroporation. *Pharm Res* 1994;11:1657–62.
- Zhao YL, Murthy SN, Manjili MH, Guan LJ, Sen A, Hui SW. Induction of cytotoxic T-lymphocytes by electroporation-enhanced needle-free skin immunization. *Vaccine* 2006;24:1282–90.
- Laddy DJ, Yan J, Khan AS, Andersen H, Cohn A, Greenhouse J, et al. Electroporation of synthetic DNA antigens offers protection in nonhuman primates challenged with highly pathogenic avian influenza virus. *J Virol* 2009;83:4624–30.
- Chiarella P, Fazio VM, Signori E. Application of electroporation in DNA vaccination protocols. *Curr Gene Ther* 2010;10:281–6.
- Pillai O, Panchagnula R. Transdermal iontophoresis of insulin. V. Effect of terpenes. *J Control Release* 2003;88:287–96.
- Chang SL, Hofmann GA, Zhang L, Defetos LJ, Banga AK. Transdermal iontophoretic delivery of salmon calcitonin. *Int J Pharm* 2000;200:107–13.
- Davarian S, Kalantari KK, Rezasoltani A, Rahimi A. Effect and persistency of botulinum toxin iontophoresis in the treatment of palmar hyperhidrosis. *Australas J Dermatol* 2008;49:75–9.
- Denet AR, Ucakar B, Pr at V. Transdermal delivery of timolol and atenolol using electroporation and iontophoresis in combination: a mechanistic approach. *Pharm Res* 2003;20:1946–51.
- Polat BE, Blankschtein D, Langer R. Low-frequency sonophoresis: application to the transdermal delivery of macromolecules and hydrophilic drugs. *Expert Opin Drug Deliv* 2010;7:1415–32.
- Dahlan A, Alpar HO, Stickings P, Sesardic D, Murdan S. Transcutaneous immunisation assisted by low-frequency ultrasound. *Int J Pharm* 2009;368:123–8.
- Dahlan A, Alpar HO, Murdan S. An investigation into the combination of low frequency ultrasound and liposomes on skin permeability. *Int J Pharm* 2009;379:139–42.
- Tezel A, Paliwal S, Shen Z, Mitragotri S. Low-frequency ultrasound as a transcutaneous immunization adjuvant. *Vaccine* 2005;23:3800–7.
- Hingson RA, Davis HS, Bloomfield RA, Brailey RF. Mass inoculation of the Salk polio vaccine with the multiple dose jet injector. *GP* 1957;15:94–6.
- Kelly K, Loskutov A, Zehring D, Puaa K, LaBarre P, Muller N, et al. Preventing contamination between injections with multiple-use nozzle needle-free injectors: a safety trial. *Vaccine* 2008;26:1344–52.
- Jackson LA, Austin G, Chen RT, Stout R, DeStefano F, Gorse GJ, et al. Vaccine Safety Datalink Study Group. Safety and immunogenicity of varying dosages of trivalent inactivated influenza vaccine administered by needle-free jet injectors. *Vaccine* 2001;19:4703–9.
- Kanazawa T, Takashima Y, Tamura T, Tsuchiya M, Shibata Y, Udagawa H, et al. Local gene expression and immune responses of vaginal DNA vaccination using a needle-free injector. *Int J Pharm* 2010;396:11–6.
- Simon JK, Carter M, Pasetti MF, Szein MB, Kotloff KL, Weniger BG, et al. Safety, tolerability, and immunogenicity of inactivated trivalent seasonal influenza vaccine administered with a needle-free disposable-syringe jet injector. *Vaccine* 2011;29:9544–50.
- Frech SA, Dupont HL, Bourgeois AL, McKenzie R, Belkind-Gerson J, Figueroa JF, et al. 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;371:2019–25.
- Etchart N, Hennino A, Friede M, Dahel K, Dupouy M, Goujon-Henry C, et al. Safety and efficacy of transcutaneous vaccination using a patch with the live-attenuated measles vaccine in humans. *Vaccine* 2007;25:6891–9.
- Glenn GM, Taylor DN, Li X, Frankel S, Montemarano A, Alving CR. Transcutaneous immunization: a human vaccine delivery strategy using a patch. *Nat Med* 2000;6:1403–6.
- Naito S, Maeyama J, Mizukami T, Takahashi M, Hamaguchi I, Yamaguchi K. Transcutaneous immunization by merely prolonging the duration of antigen presence on the skin of mice induces a potent antigen-specific antibody response even in the absence of an adjuvant. *Vaccine* 2007;25:8762–70.
- McKenzie R, Bourgeois AL, Frech SA, Flyer DC, Bloom A, Kazempour K, et al. 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;25:3684–91.
- [58] Ishii Y, Nakae T, Sakamoto F, Matsuo K, Matsuo K, Quan YS, et al. Transcutaneous vaccination system using a hydrogel patch for viral and bacterial infection. *J Control Release* 2008;131:113–20.
- [59] Matsuo K, Ishii Y, Quan YS, Kamiyama F, Mukai Y, Yoshioka Y, et al. Transcutaneous vaccination using a hydrogel patch induces effective immune responses to tetanus and diphtheria toxoid in hairless rat. *J Control Release* 2011;149:15–20.
- [60] Matsuo K, Ishii Y, Quan YS, Kamiyama F, Mukai Y, Okada N, et al. Characterization of transcutaneous protein delivery by a hydrogel patch in animal, human, and tissue-engineered skin models. *Biol Pharm Bull* 2011;34:586–9.
- [61] Matsuo K, Ishii Y, Quan YS, Kamiyama F, Asada H, Mukai Y, et al. Compositional optimization and safety assessment of a hydrogel patch as a transcutaneous immunization device. *Biol Pharm Bull* 2011;34:1835–40.
- [62] Hirobe S, Matsuo K, Quan YS, Kamiyama F, Morito H, Asada H, et al. Clinical study of transcutaneous vaccination using a hydrogel patch for tetanus and diphtheria. *Vaccine* 2012;30:1847–54.
- [63] Edelberg R. Relation of electrical properties of skin to structure and physiologic state. *J Invest Dermatol* 1977;69:324–7.
- [64] Alonso A, Meirelles NC, Yushmanov VE, Tabak M. Water increases the fluidity of intercellular membranes of stratum corneum: correlation with water permeability, elastic, and electrical resistance properties. *J Invest Dermatol* 1996;106:1058–63.
- [65] Vogt A, Combadière B, Hadam S, Stieler KM, Lademann J, Schaefer H, et al. Blume-Peytavi U. 400 nm, but not 750 or 1,500 nm, nanoparticles enter epidermal CD1a⁺ cells after transcutaneous application on human skin. *J Invest Dermatol* 2006;126:1316–22.
- [66] Jung S, Otberg N, Thiede G, Richter H, Sterry W, Panzner S, et al. Innovative liposomes as a transfollicular drug delivery system: penetration into porcine hair follicles. *J Invest Dermatol* 2006;126:1728–32.
- [67] Baroli B, Ennas MG, Loffredo F, Isola M, Pinna R, López-Quintela MA. Penetration of metallic nanoparticles in human full-thickness skin. *J Invest Dermatol* 2007;127:1701–12.
- [68] Henry S, McAllister DV, Allen MG, Prausnitz MR. Microfabricated microneedles: a novel approach to transdermal drug delivery. *J Pharm Sci* 1988;77:922–5.
- [69] Park JH, Allen MG, Prausnitz MR. Biodegradable polymer microneedles: fabrication, mechanics and transdermal drug delivery. *J Control Release* 2005;104:51–66.
- [70] Vrdoljak A, McGrath MG, Carey JB, Draper SJ, Hill AV, O'Mahony C, et al. Coated microneedle arrays for transcutaneous delivery of live virus vaccines. *J Control Release* 2012;159:34–42.
- [71] Hiraishi Y, Nandakumar S, Choi SO, Lee JW, Kim YC, Posey JE, et al. Bacillus Calmette-Guérin vaccination using a microneedle patch. *Vaccine* 2011;29:2626–36.
- [72] Sullivan SP, Koutsouanos DG, Del Pilar Martin M, Lee JW, Zarnitsyn V, Choi SO, et al. Dissolving polymer microneedle patches for influenza vaccination. *Nat Med* 2010;16:915–20.
- [73] Kim YC, Quan FS, Yoo DG, Compans RW, Kang SM, Prausnitz MR. Improved influenza vaccination in the skin using vaccine coated microneedles. *Vaccine* 2009;27:6932–8.
- [74] Ke CJ, Lin YJ, Hu YC, Chiang WL, Chen KJ, Yang WC, et al. Multidrug release based on microneedle arrays filled with pH-responsive PLGA hollow microspheres. *Biomaterials* 2012;33:5156–65.
- [75] Gerstel MS, Place VA. Drug Delivery Device, US Patent No. 3 1976; 964: 482.
- [76] Matsuo K, Yokota Y, Zhai Y, Quan YS, Kamiyama F, Mukai Y, et al. Low-invasive and effective transcutaneous immunization system using a novel dissolving microneedle array for soluble and particulate antigens. *J Control Release* 2012;161:10–7.
- [77] Matsuo K, Hirobe S, Yokota Y, Ayabe Y, Seto M, Quan YS, et al. Transcutaneous immunization using a dissolving microneedle array protects against tetanus, diphtheria, malaria, and influenza. *J Control Release* 2012;160:495–501.
- [78] Hiraishi Y, Nakagawa T, Quan YS, Kamiyama F, Hirobe S, Okada N, et al. Performance and characteristics evaluation of a sodium hyaluronate-based microneedle patch for a transcutaneous drug delivery system. *Int J Pharm* 2013;441:570–9.
- [79] Hansen S, Lehr CM. Nanoparticles for transcutaneous vaccination. *Microb Biotechnol* 2012;5:156–67.
- [80] Kohli AK, Alpar HO. Potential use of nanoparticles for transcutaneous vaccine delivery: effect of particle size and charge. *Int J Pharm* 2004;275:13–7.
- [81] Mattheolabakis G, Lagoumintzis G, Panagi Z, Papadimitriou E, Partidos CD, Avgoustakis K. Transcutaneous delivery of a nanoencapsulated antigen: induction of immune responses. *Int J Pharm* 2010;385:187–93.
- [82] Prego C, Paolicelli P, Díaz B, Vicente S, Sánchez A, González-Fernández A, et al. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. *Vaccine* 2010;28:2607–14.
- [83] Lee PW, Peng SF, Su CJ, Mi FL, Chen HL, Wei MC, et al. The use of biodegradable polymeric nanoparticles in combination with a low-pressure gene gun for transdermal DNA delivery. *Biomaterials* 2008;29:742–51.
- [84] Li N, Peng LH, Chen X, Nakagawa S, Gao JQ. Effective transcutaneous immunization by antigen-loaded flexible liposome in vivo. *Int J Nanomedicine* 2011;6:3241–50.
- [85] Paul A, Cevc G, Bachhawat BK. Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transferosomes. *Vaccine* 1998;16:188–95.
- [86] Mishra D, Dubey V, Asthana A, Saraf DK, Jain NK. Elastic liposomes mediated transcutaneous immunization against Hepatitis B. *Vaccine* 2006;24:4847–55.
- [87] Ding Z, Bivas-Benita M, Hirschberg H, Kersten GF, Jiskoot W, Bouwstra JA. Preparation and characterization of diphtheria toxoid-loaded elastic vesicles for transcutaneous immunization. *J Drug Target* 2008;16:555–63.
- [88] Mishra V, Mahor S, Rawat A, Dubey P, Gupta PN, Singh P, et al. Development of novel fusogenic vesosomes for transcutaneous immunization. *Vaccine* 2006;24:5559–70.
- [89] Wang J, Hu JH, Li F, et al. Strong cellular and humoral immune responses induced by transcutaneous immunization with HBsAg DNA-cationic deformable liposome complex. *Exp Dermatol* 2007;16:724–9.
- [90] Combadière B, Vogt A, Mahé B, Costagliola D, Hadam S, Bonduelle O, et al. Preferential amplification of CD8 effector-T cells after transcutaneous application of an inactivated influenza vaccine: a randomized phase I trial. *PLoS ONE* 2010;5:e10818.
- [91] Van Damme P, Oosterhuis-Kafeja F, Van der Wielen M, Almagor Y, Sharon O, Levin Y. Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults. *Vaccine* 2009;27:454–9.
- [92] Pettis RJ, Harvey AJ. Microneedle delivery: clinical studies and emerging medical applications. *Ther Deliv* 2012;3:357–71.
- [93] Birchall JC, Clemo R, Anstey A, John DN. Microneedles in clinical practice – an exploratory study into the opinions of healthcare professionals and the public. *Pharm Res* 2011;28:95–106.
- [94] Belshe RB, Newman FK, Cannon J, Duane C, Treanor J, Van Hoek C, et al. Serum antibody responses after intradermal vaccination against influenza. *N Engl J Med* 2004;351:2286–94.
- [95] Kenney RT, Frech SA, Muenz LR, Villar CP, Glenn GM. Dose sparing with intradermal injection of influenza vaccine. *N Engl J Med* 2004;351:2295–301.