

# Mucosal Immune Responses Induced by Transcutaneous Vaccines

L. B. Lawson, J. D. Clements and L. C. Freytag

**Abstract** The skin has been investigated as a site for vaccine delivery only since the late 1990s. However, much has been discovered about the cell populations that reside in the skin, their active role in immune responses, and the fate of transcutaneously applied antigens. Transcutaneous immunization (TCI) is a safe, effective means of inducing immune responses against a number of pathogens. One of the most notable benefits of TCI is the induction of immune responses in both systemic and mucosal compartments. This chapter focuses on the transport of antigen into and beyond intact skin, the cutaneous sentinel cell populations that play a role in TCI, and the types of mucosal immune responses that have been generated. A number of in vivo studies in murine models have provided information about the broad responses induced by TCI. Cellular and humoral responses and protection against challenge have been noted in the gastrointestinal, reproductive, and respiratory tracts. Clinical trials have demonstrated the benefits of this vaccine delivery route in humans. As with other routes of immunization, the type of vaccine formulation and choice of adjuvant may be critical for achieving appropriate responses and can be tailored to activate specific immune-responsive cells in the skin to increase the efficacy of TCI against mucosal pathogens.

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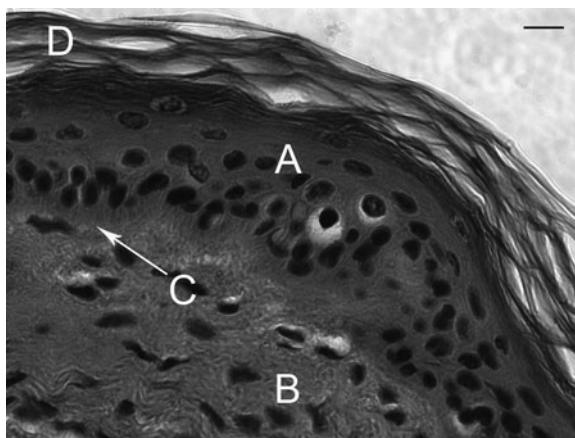
## 1 Introduction

Transcutaneous immunization (TCI) also known as transdermal vaccine delivery (TVD) is a novel, safe, non-invasive method of inducing antigen-specific cellular and humoral immune responses via direct application of a vaccine antigen onto the skin. The advantages of needle-free immunization include easier and faster vaccine administration, increased safety, elimination of pain at the injection site, reduction of trained healthcare personnel, and improved compliance with vaccination schedules. The skin, like other epithelial surfaces, serves a crucial function in regulating exchange of matter between the body and the outside environment. The skin's role as a barrier against moisture loss, chemical or physical injury and pathogen entry has been extensively studied. However, only in the past few decades, the active role of immune-responsive cells of the skin has been noted (Silberberg-Sinakin et al. 1976; Sauder 1983; Streilin 1985). More recently, the concept of using the skin as a site of vaccine administration has been explored; the first investigation of topical vaccination was reported in 1997 (Tang et al. 1997). Since then, a substantial number of published reports have demonstrated the feasibility of TCI as an effective vaccine delivery method in animals and humans. It has also become evident that a key player in the outcome of TCI is the choice of adjuvant employed. The induction of humoral and cellular responses in both systemic and mucosal compartments, first reported in 1998 (Glenn et al. 1998b), is one of the most notable benefits of this technique over traditional parenteral vaccine administration.

## 2 Transport of Antigen Through the Skin

The skin is composed of three major layers: the epidermis, dermis, and hypodermis or subcutis (Young et al. 2006). Figure 1 displays a micrograph of the structure of the outermost layers of the skin; cells that are most involved in transcutaneous

**Fig. 1** Hematoxylin and eosin stain of porcine skin section showing the major tissue layers, the epidermis (A) and dermis (B), with the basement membrane (C) located at the junction between the two. The outermost region of the epidermis is the stratum corneum (D). Bar = 10  $\mu$ m. Reprinted with permission from Macmillan Publishers Ltd: Clinical Pharmacology & Therapeutics (Lawson et al. 2007)



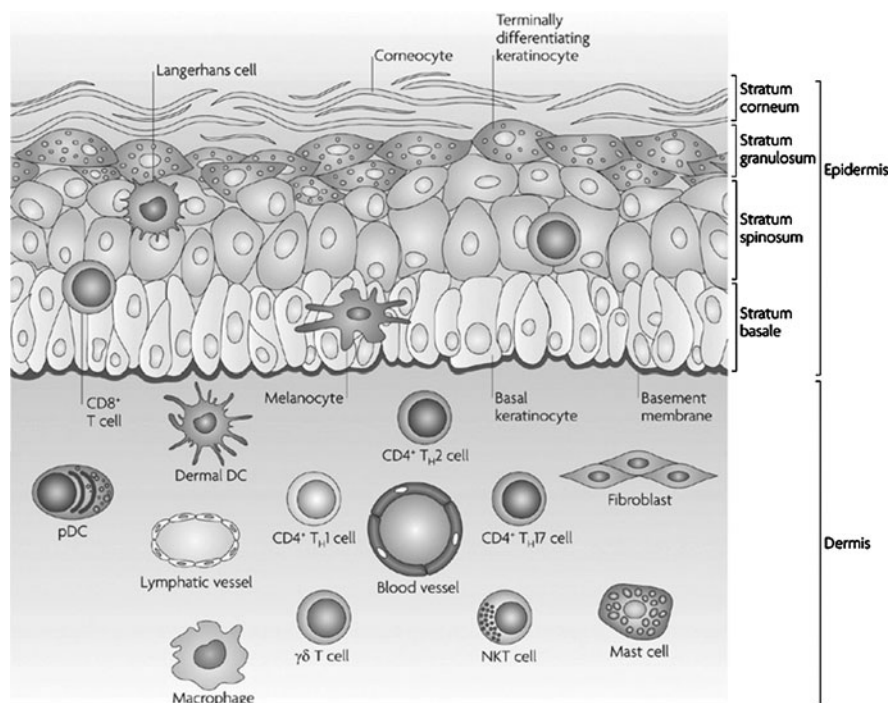
immune response are located within the epidermal and dermal layers. To achieve an efficacious immune response, antigens applied to the skin must traverse the topmost keratinized layers of the skin to reach the immune-responsive cells. The outer portion of the epidermis, the stratum corneum, is composed of non-nucleated highly keratinized cells surrounded by densely packed lipid molecules. This layer provides a barrier to moisture loss and pathogen entry, and also limits the permeation of large molecular-weight antigens. Various techniques have been considered to circumvent this limitation for TCI, including the use of adjuvants, disrupting the integrity of the stratum corneum by tape stripping, swabbing with alcohol or other solvents, hydration, ultrasound, microneedles, and other physical or chemical permeation enhancers. Tape stripping and solvent application not only abrogate the skin barrier but also activate resident cells to augment expression of cytokine and co-stimulatory molecules and to enhance antigen presentation (Nickoloff and Naidu 1994; Nishijima et al. 1997). While such methods can increase antigen permeation and typically improve immune response, transport of highly immunogenic antigens and adjuvants across intact skin with induction of protective immunity has also been demonstrated (Glenn et al. 1998a, b).

Another consideration for TCI is the anatomical site of antigen application. It is known that variations in the thickness and composition of the skin exist at different sites of the human body, indicating potential differences in permeability of applied antigens. A few studies have compared the efficacy of TCI at different anatomical sites in mice. Although there were differences in the role of skin-resident dendritic cells (DCs) following TCI on the ear versus the flank, no differences in T cell proliferation in the draining lymph nodes were observed (Wang et al. 2008). There were also no significant differences in serum antibody levels following ear or back TCI (Scharton-Kersten et al. 1999). However, there were greater serum antibody responses with administration of an HIV peptide and cholera toxin (CT) adjuvant on the back and abdomen compared to administration on the ear (Belyakov et al. 2004). Antigen-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) in the gut mucosa

were also lower with immunization on the ear than on the back or abdomen (Belyakov et al. 2004). In clinical trials, transcutaneous vaccines have been administered on the upper arm (Glenn et al. 2000; Frech et al. 2008) and forearm (Etchart et al. 2007) of volunteers.

### 3 Immune-Responsive Cells in the Skin

The skin is more than a passive barrier protecting the host against physical or chemical damage. We now know that non-inflamed skin is an immunologically active site that contains numerous cell populations of immune-responsive cells. The presence and function of these cells determines the response to antigens that permeate across the stratum corneum. Figure 2 is a schematic representation of the stratified layers of the epidermis, dermis and the main cell types involved in immune surveillance, antigen uptake, and initiation of immune responses. Contributors to the cutaneous immune response include keratinocytes, epidermal and



**Fig. 2** Schematic representation of the skin anatomy and the cellular effectors involved in the generation of immune responses. Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Nestle et al. 2009)

dermal DCs (dDCs), T lymphocytes, Natural Killer (NK)-T cells, mast cells and macrophages, among others. Curiously, the presence of B cells, B cell follicles, or germinal centers seen in some mucosal tissues has not been noted in the skin (Kapsenberg and Bos 1998).

### **3.1 Keratinocytes**

Comprising 95% of epidermal cells (Wood et al. 1992), keratinocytes are epithelial cells that are predominant in the epidermis and are responsible for the production of keratin, a fibrous protein that contributes to the skin's protective barrier by providing structural support (Young et al. 2006). Through their production of various cytokines and chemokines, keratinocytes have a crucial role in maintaining the skin's physical barrier structure as well as in contributing to local innate and acquired immune responses. Cytokine and chemokine production by keratinocytes occurs both constitutively and in response to various stimuli. Disruption of the epidermal barrier can induce cytokine production by keratinocytes (Wood et al. 1992). Activation is also triggered by proinflammatory cytokines produced by other cells (Williams and Kupper 1996). Early in the immune response, cytokine production by keratinocytes induces motility of antigen presenting cells (APCs) in the skin (Kissenpfennig and Malissen 2006). Indicating their role in innate immune response, human keratinocytes express functional Toll-like receptors (TLRs) which contribute to the immune response against viral and bacterial pathogens (Köllisch et al. 2005; Kalali et al. 2008). TLR activation of keratinocytes has been shown to enhance DC activation in vitro (Sugita et al. 2007). Recent publications suggest that keratinocytes may also play a role as non-professional APCs because they express MHC class II molecules and can present peptide antigens to CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Black et al. 2007). Keratinocytes play a role in directing the cutaneous immune response toward a cellular or humoral response, in the regulation of the thickness of the epidermis, and in promoting growth, maturation, and mobilization of leukocytes from the blood (Williams and Kupper 1996).

### **3.2 Dendritic Cells**

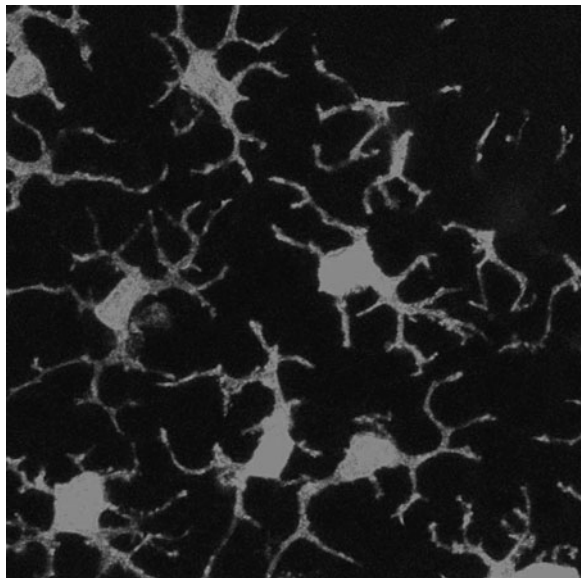
The skin is rich in APCs such as macrophages and DCs, which act as sentinels patrolling the skin and are an essential component of innate defense against pathogens. While macrophages mostly function in pathogen clearance, DCs specialize in linking innate and adaptive immune responses and their primary role is antigen presentation rather than pathogen elimination. DCs are professional APCs, capable of efficiently capturing, processing, and presenting antigens on their surfaces in MHC molecules in close proximity to co-stimulatory molecules. DCs

are especially potent at priming naive T cells to initiate antigen-specific immune responses.

Two types of DCs—dDCs and Langerhans cells (LCs)—are abundant in the skin of humans and animals. Upon stimulation, dDCs and LCs initiate antigen uptake, differentiate, become more mature, and migrate via afferent lymphatics to the draining lymph nodes, where they contact naive T and B cells. Within the epidermis, LCs play a critical role in antigen presentation. LCs comprise approximately 2% of the total epidermal cell population; however, these cells cover over 25% of the total surface area of the skin, as illustrated in Fig. 3. Their orientation parallel to the skin and dendrites maximizes exposure to foreign antigens that enter the skin (Yu et al. 1994). LCs typically localize within the upper level of the viable epidermis, although some are found deeper toward the basement membrane (Hauser et al. 1991). Most early studies of LC were focused on their role in contact hypersensitivity (Silberberg-Sinakin et al. 1976). Their role in systemic immune responses is now becoming more clearly understood.

A distinguishing characteristic of LCs is the presence of cytoplasmic Birbeck granules. The development of these tennis racket-shaped organelles is dependent on the expression of the endocytic receptor langerin/CD207. Once thought to be another defining characteristic of LC, langerin expression was recently shown not only in LCs but also in other distinct DC populations of the dermis (Bursch et al. 2007). Bone marrow-derived circulating DC can also express langerin (Chang et al. 2008). Another distinguishing molecule expressed by LCs is CD1a. This molecule is associated with the ability of LCs to present certain bacterial lipids and glycolipids as well as peptide antigen to T cells (Kapsenberg and Bos 1998).

**Fig. 3** Confocal micrograph showing the dense network of epidermal Langerhans cells in the ear of mice expressing enhanced green fluorescence protein under control of the *langerin* gene. Reprinted from Trends in Immunology (Kissenpfennig and Malissen 2006) with permission from Elsevier



Langerhans cells upregulate MHC class II molecules, co-stimulatory molecule CD40, and lymph node homing molecule CCR7 during migration from the skin to draining lymph nodes (Merad et al. 2008).

Like LCs, dDCs are active immune responders. dDCs were identified much more recently than their epidermal counterparts and are not as well characterized as LCs (Romani et al. 2006). dDCs are more abundant than LCs, and they have a distinct role in immune responses (Nestle et al. 1998). Expression of CD11c distinguishes dDCs from LCs. The CD1c (blood DC antigen-1) molecule is also expressed by dDCs but not LCs (Zaba et al. 2007). dDCs are typically localized in the perivascular region of the dermis (Nestle et al. 1998).

dDCs and LCs are comparable in their ability to present soluble antigen to T cells in vitro (Nestle et al. 1998). However, these two cell populations have distinguishing TLR expression profiles, indicating differences in their role in immune responses. dDCs express TLRs 1–8, while LCs have impaired expression of TLR2, TLR4, TLR5, and TLR8. As a result, LCs are less reactive to Gram-negative and Gram-positive bacteria, which likely prevents LCs from initiating an inflammatory response against commensal bacteria colonizing the skin. Langerhans cells and dDCs do react comparably to viral antigens (van der Aar et al. 2007). The TLR expression profile of LCs and dDCs is an important consideration in designing vaccine formulations for TCI.

### 3.3 *T Cells*

Substantial numbers of T lymphocytes exist in the human skin with limited distribution in the epidermis and a more prevalent population in the dermal perivascular region (Spetz et al. 1996; Clark et al. 2006). The T cells in non-inflamed skin express cutaneous-lymphocyte-associated antigen (CLA), which is a ligand for E-selectin. Interaction of CLA<sup>+</sup> T cells with E-selectin in the cutaneous endothelium facilitates selective migration of lymphocytes to the skin. Various adhesion molecules, leukocyte chemoattractants and cell surface markers have also been implicated in the homing of T cells to the skin. In this context, an interesting recent observation indicates that Vitamin D<sub>3</sub>, generated in the skin upon exposure to sunlight, can be converted to its active form (1,25(OH)<sub>2</sub>D<sub>3</sub>) by subsets of DC, macrophages and keratinocytes. This activated form has been shown to induce T cell expression of CCR10 chemokine receptor and migration toward keratinocyte-produced CCL27 in vitro (Sigmundsdottir et al. 2007). Regardless of the mechanisms governing T cell homing to the skin, it is evident that large pools of T cells are continuously present in the cutaneous environment, where they can immediately contact antigen-capturing DCs.

Although resident skin T cells are phenotypically diverse, most are Th1 effector memory cells, with lesser numbers of Th2 cells, central memory cells and regulatory T cells. Epidermal T cells are found in close proximity to LCs in the basal keratinocyte layer. The majority of epidermal T lymphocytes are CD8<sup>+</sup>  $\alpha\beta$ . In



contrast, CD4<sup>+</sup> memory T cells predominate in the perivascular dermis (Kapsenberg and Bos 1998; Clark et al. 2006). Dermal T cells localize close to postcapillary venules in the epidermal–dermal junction (Fig. 2). Several subsets of CD4<sup>+</sup> Th1, Th2 and Th17 are found in the dermis. These have been associated with inflammatory skin diseases, but it is likely that they also participate in immune surveillance and immune responses to antigens encountered by APCs in the skin.

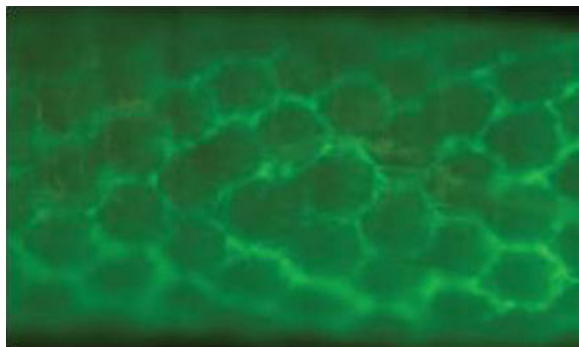
### 3.4 Other Immune-Responsive Cells

Other cell populations in the skin contribute to the outcome of TCI. Heib et al. 2007 demonstrated a role for dermal mast cells in LC migration, inflammation, and in the induction of CTL responses following TCI. A population of macrophages has been identified in the dermis of normal human skin based on expression of CD163, a scavenger receptor selectively expressed on monocytes and macrophages. These cells appear to be weak stimulators of T cell proliferation (Zaba et al. 2007). The roles of macrophages, granulocytes, NK cells, and  $\gamma\delta$  T cells in skin immune responses have not been elucidated, but this information would be helpful for designing and formulating transcutaneous vaccines such that they generate desired immune responses (Warger et al. 2007).

## 4 Antigen Migration from the Skin

Running parallel to the skin surface, a network of lymphatic vessels exists just below the epidermis. These vessels form an interconnected mesh-like network (see Fig. 4) and drain through slightly larger ducts to collecting vessels in the deeper regions of the dermis. The collecting vessels actively propel lymphatic fluid from

**Fig. 4** Fluorescence micrograph showing the regular polygonal pattern of skin lymphatic vessels using fluorescent lymphatic tracers. Microscopic view is through the epidermis of a mouse tail. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Randolph et al. 2005)





the tissue to the lymph nodes. Flow in these vessels is unidirectional such that cells and molecules downstream in the lymph nodes do not return to or directly influence cells in the periphery (Randolph et al. 2005).

The migration of transcutaneously applied material has been studied using fluorescent dyes, fluorescently labeled proteins, and electron microscopy to better understand the responses induced by this immunization route. Fluorescent dye applied to the skin with or without protein immunogen was found in the regional draining, but not distal, lymph nodes of mice 24 h after administration. In the presence of protein immunogen, an increase in DC activation markers in the lymph nodes most proximal to the site of transcutaneous application was noted (Guebre-Xabier et al. 2003). In early investigations, ferritin-bearing LCs in draining lymph nodes were observed as soon as 4 h after the application of this protein on skin (Silberberg-Sinakin et al. 1976).

The use of knock-in mice expressing green fluorescent protein (GFP) under the control of the *langerin* gene has allowed researchers to distinguish trafficking of LC and other langerin<sup>+</sup> cells from that of dDC. dDC arrived in the lymph nodes 24 h post-treatment, while GFP-laden LCs were not detected until later time points (Kissenpfennig et al. 2005). Using similar techniques, the relative migration of LCs versus other APCs was shown to vary depending on the site (e.g. ear or flank) of TCI in mice (Wang et al. 2008). Together, these studies have demonstrated an active role for LC and other cell populations in the transport of antigens to draining lymph nodes following TCI.

When fluorescently labeled *E. coli* heat labile enterotoxin (LT) was applied to the skin of mice, the fluorescent label was detected in APCs in intestinal Peyer's patches but not in the spleen or non-draining lymph nodes at 24 and 48 h, suggesting that APCs migrate from the skin to directly present antigen to intestinal lymphocytes (Belyakov et al. 2004). Whether these skin-derived APCs reach other mucosal locations has not yet been determined.

## 5 Adjuvants for Transcutaneous Vaccines

A critical element in enhancing antigen-specific responses by TCI is the choice of method used to activate APCs in the skin. Different strategies have been employed to enhance the immune response, including disruption of the stratum corneum (by tape stripping, microneedles, electrical impulses, prolonged hydration), micro- or nanocarriers, and the use of adjuvants such as imiquimod, CpG, or bacterial enterotoxins (Partidos et al. 2004; Glenn and Kenney 2006).

The bacterial ADP-ribosylating enterotoxins (BARE)—CT, produced by various strains of *Vibrio cholerae*, and the closely related LT, produced by some enterotoxigenic strains of *Escherichia coli*—are effective adjuvants for systemic, mucosal and transdermal vaccines (Clements et al. 1988; Dickinson and Clements 1996; Glenn et al. 1998a; Freytag and Clements 1999, 2005). Both CT and LT are synthesized as multisubunit toxins with A and B components. The A-subunit is the

enzymatically active moiety and consists of two chains, A1 and A2, joined by a proteolytically sensitive link (Arg192) subtended by a disulfide loop. Like other A–B toxins, CT and LT require nicking and disulfide reduction to be fully biologically active. When CT or LT first encounter a mammalian cell, they bind to the surface through interaction of the B-subunit pentamer. The A2 peptide of CT or LT facilitates association of A1 with the B-pentamer and helps direct retrograde transport of these molecules through the Golgi cisternae to the ER. Once in the ER, the A1 chain is transported across the membrane into the cytosol where it binds  $\text{NAD}^+$  and transfers the ADP-ribose moiety from  $\text{NAD}^+$  to the  $\alpha$  subunit of one member of the heterotrimeric GTP-binding protein family. As a consequence, adenylate cyclase is irreversibly activated, leading to elevation in intracellular cyclic AMP (cAMP). Increased levels of cAMP activate protein kinase A which phosphorylates and opens the cystic fibrosis transmembrane conductance regulator chloride channel. Chloride efflux results in the concomitant osmotic movement of water into the gut lumen and the profuse watery diarrhea characteristic of cholera or enterotoxigenic *E. coli* infection in humans.

It has been reported that CT upregulates expression of co-stimulatory molecules on DC and promotes DC mobility, maturation and activation (Gagliardi et al. 2000). However, the exact mechanisms responsible for the adjuvant effects of BARE on DC have not been identified. The BARE can elicit mixed Th1/Th2 or Th2-biased immune responses, which may depend on the levels of cAMP induced in cells as cAMP ultimately suppresses DC production of IL-12 by inhibiting activity of the interferon regulatory factor 8 transcription factor (la Sala et al. 2010). Only a few studies have addressed the effects that CT and LT exert on skin-derived APCs. One suggested that CT adjuvant activity after skin immunization may be due to the secretion of proinflammatory cytokines from activated epidermal LCs and keratinocytes (Partidos et al. 2004). It has also been proposed that apoptosis of keratinocytes creates intercellular spaces resulting in more efficient diffusion of adjuvant and antigen molecules (Partidos et al. 2004).

Non-methylated CpG oligodeoxynucleotides (CpG ODNs) are TLR9 agonists. The adjuvant activity of CpG ODN has been attributed to several different effects on innate and adaptive immune responses. First, CpG ODNs cause B cells to proliferate and secrete immunoglobulin, synergizing strongly with antigen-specific effects mediated through B cells. In addition, CpG ODNs upregulates expression of co-stimulatory molecules and MHC class II molecules, improving antigen presentation. CpG ODNs also directly activate monocytes, macrophages and DC to secrete  $\text{IFN-}\alpha/\beta$ , IL-6, IL-12, GM-CSF, chemokines, and  $\text{TNF-}\alpha$ , which in turn stimulate T cells to secrete additional cytokines and NK cells to secrete  $\text{IFN-}\gamma$ . A T-helper function is provided by the strong Th1-like pattern of cytokine production that is dominated by IL-12 and  $\text{IFN-}\gamma$ , with little secretion of Th2 cytokines. The exact mechanisms responsible for the immunostimulatory action of CpG ODN are only now being resolved. One hypothesis is that CpG-DNA (either as a free molecule or encapsulated in whole bacteria) is taken up by an APC. After processing through a chloroquine-sensitive pathway, signaling is triggered by engagement of TLR9. The proteins myeloid differentiation factor 88 (MyD88),

interleukin-1 receptor-associated kinase and tumor necrosis factor receptor-associated factor 6 activate cellular kinases such as I $\kappa$ B kinase and mitogen-activated protein kinase. Signal transduction through these well-known pathways leads to gene induction and evokes effector functions, such as cytokine secretion. CpG ODNs have been shown to function as effective adjuvants for transdermally delivered vaccines (Klimuk et al. 2004; Sugita et al. 2007).

Imiquimod (1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine) is a synthetic derivative of imidazoquinoline, which is a ligand for TLR7. Imiquimod (marketed as Aldara<sup>TM</sup>) is an FDA-approved therapy for the treatment of human external genital warts, actinic keratosis, and basal cell carcinoma. This compound acts as an immune modifier and activates DCs in vitro by inducing MyD88-dependent DC maturation and release of inflammatory cytokines. Rechtsteiner et al. (2005) recently described a study in which skin application of a CTL epitope in combination with imiquimod induced a robust CTL response against the target characterized by T cell proliferation, cytolytic activity and IFN- $\gamma$  production. Topical imiquimod application to mouse skin induces migration of LCs from the epidermis to the local lymph nodes. It has been demonstrated that imiquimod also induces maturation of human epidermal LCs, enhances IL-12 production, and increases IFN- $\alpha$  production by CD4<sup>+</sup> T cells (Burns et al. 2000). It is thought that the effects of imiquimod on epidermal LCs are an important mechanism of action by which imiquimod induces Th1-dominant cellular responses in situ during the treatment of certain malignancies. Although it is known that dDCs, LCs and keratinocytes express intracytoplasmic TLR7, the exact effects of imiquimod on the activation of skin-derived APCs have not been defined.

Various nanocarriers and specifically tailored formulations show promise for TCI either by enhancing permeation through the stratum corneum or by increasing antigen uptake by APCs and other cells. The use of a lipid matrix for transcutaneous delivery of a *Helicobacter pylori* vaccine in mice induced protection against gastric challenge in the absence of co-administered adjuvants, although the addition of CT and CpG to the lipid formulation further enhanced protection (Hickey et al. 2009b). The use of particulate micro- and nanocarriers with encapsulated or adsorbed antigen can influence antigen uptake in the skin. The particle composition (lipid, polymer, virus-like particles, etc.), size, charge and degradability are important factors for interaction with DC, T cells, B cells and other components of the immune response. Mishra et al. (2006) demonstrated increased ex vivo macrophage uptake and in vivo lymphatic accumulation, follicular enlargement and both serum and salivary IgA responses with elastic liposomes applied transcutaneously. This supports the role of these and other carriers in modulating the immune response upon TCI.

Overall, it is clear that adjuvants play a fundamental role in the activation of skin DC upon transcutaneous delivery of vaccines. It is critical to design studies to understand how different adjuvants act upon skin APCs to influence the ultimate outcome of T cell activation. Deciphering the effects of adjuvants on skin APC maturation and activation can lead to the development of tailored vaccines for specific pathogens and specific immune responses.

## 6 Mucosal Immune Responses to Transcutaneous Antigens in Murine Models

TCI achieved by direct topical application of a vaccine preparation is thought to work because the vaccine components permeate the skin and interact directly with APC in the epidermis and dermis, which then migrate and interact with naive T cells in the lymph nodes to initiate adaptive immune responses (Kripke et al. 1990). Many published reports and results of clinical trials have demonstrated that TCI can induce robust systemic and mucosal immune responses in animals and humans (Yu et al. 2002; Glenn et al. 2007; Uddowla et al. 2007; Vogt et al. 2008).

### 6.1 Mucosal Antibody Responses in Mice Following TCI

Induction of antigen-specific antibody responses in mucosal tissues after TCI has been amply documented in animal models, particularly in mice. IgG and IgA antibodies against the immunizing antigen have been observed in the gastrointestinal, respiratory and genitourinary tracts. The mechanisms involved in the regulation of these responses are not well understood, but recent studies have documented the migration of activated DCs from the skin to the gut mucosa. Chang et al. 2008 found antigen-specific IgG and IgA antibody-secreting cells (ASC) in the small and large intestine after TCI with tetanus toxoid (TT) and CT but not after subcutaneous or intraperitoneal immunization. Others have also reported induction of specific antibodies in the intestine after TCI with various combinations of antigen and adjuvant. Among these studies, some significant observations include the generation of fecal anti-OVA IgA and IgG (Naito et al. 2007), anti-*E. coli* CS6 IgA and IgG (Yu et al. 2002) and anti-*H. pylori* IgA (Hickey et al. 2009b).

The use of TCI to protect against pathogens associated with periodontitis has been demonstrated. TCI of mice with a *Porphyromonas gingivalis* protein immunogen and CT adjuvant induced antigen-specific IgA and IgG ASC in spleens. However, ASC were present in much lower numbers in the salivary glands (Maeba et al. 2005). In long-term studies, serum IgG and IgA and salivary IgG levels remained elevated 1-year post-immunization, and oral challenge with *P. gingivalis* resulted in significantly less bone loss (Ishikura et al. 2009).

The ability to induce antigen-specific ASC in the female reproductive tract of mice using TCI has also been demonstrated. In one study (Gockel et al. 2000), the numbers of ASC detected in the uterus and vagina of mice given TCI with TT and CT were found to exceed the numbers of ASC in the small intestine and salivary glands. IgG and IgA antibodies were also detected in feces, vaginal lavage, saliva, and sera of these mice. There were no mucosal or systemic IgA responses in the absence of CT adjuvant (Gockel et al. 2000). In mice immunized transcutaneously with the major outer membrane protein (MOMP) of *Chlamydia muridarum*, the number of antigen-specific ASC in vaginal tissue was increased to a greater extent

by co-delivery with CpG ODN when compared to CT (Berry et al. 2004). In another study (Skelding et al. 2006), TCI with MOMP and a mixture of CpG and CT induced antigen-specific ASC in lung tissue and protection against respiratory chlamydial challenge (Skelding et al. 2006). More recently, Hickey et al. 2009a reported that TCI with *C. muridarum* MOMP and Lipid C (a lipid-based adjuvant) or CT plus CpG was able to elicit MOMP-specific pulmonary IgG and vaginal IgG and IgA and to partially protect against chlamydia infection following vaginal or nasal challenge.

## 6.2 Cell-Mediated Immune Responses in Mice Following TCI

While induction of mucosal antibodies in response to TCI has been extensively documented, fewer studies have examined the antigen-specific cellular mucosal responses induced by this immunization strategy. Proliferation by CD8<sup>+</sup> T cells is one cellular response that has been demonstrated in in vivo murine studies (Stoitzner et al. 2006). Skin-derived DCs have been shown to play a direct role in antigen presentation after TCI with recombinant viral vectors and in the induction of CD8<sup>+</sup> T cell responses (He et al. 2006). TCI with imiquimod plus a peptide containing a T cell epitope has generated antigen-specific CTL in the spleen (Rechtsteiner et al. 2005).

Cell-mediated responses in the murine mucosa have also been noted after TCI with an HIV peptide. Following transdermal delivery of the vaccine, antigen-specific CD8<sup>+</sup> CTLs were observed in the Peyer's patches of the small intestine and in the spleen. The vaccine was adjuvanted by CT, LT or a combination of CT and CpG. This immunization strategy resulted in reduction of viral loads after intrarectal challenge of mice with recombinant vaccinia virus encoding HIV gp160 (Belyakov et al. 2004).

In mice transcutaneously immunized with *C. muridarum* MOMP and a mixture of CT and CpG, a balanced Th1/Th2 cytokine response was noted, and clearance of chlamydia infection was enhanced following intravaginal challenge (Berry et al. 2004).

With most antigens applied transcutaneously, the use of appropriate adjuvant is essential for inducing spleen and skin-draining lymph node lymphoproliferative or cytokine responses to antigen. Interestingly, TCI with CT as an adjuvant for TT induced a mixed Th1/Th2 response in contrast to the predominant Th2-type response elicited by oral or nasal immunization with CT and TT (Hammond et al. 2001). Transcutaneous application of LT adjuvant and a mutant of this protein (LT-R192G) has also induced a balanced Th1/Th2 response with robust antibody production, lymphocyte proliferation, and cytokine production in the lymph nodes (Hammond et al. 2001).

An interesting approach for increasing the efficacy of transcutaneous vaccines has been to immunize with whole organisms instead of purified subunit antigens. TCI against influenza, using formalin-inactivated influenza virus in the presence or

absence of CT, induced systemic and mucosal antibody responses as well as cytokines. Titers of hemagglutination inhibition (HAI) and neutralizing antibody were enhanced when CT was included in the vaccine preparation (Skountzou et al. 2006; Skountzou and Kang 2009). This study also revealed that TCI with inactivated influenza virus induced secretion of IL-4 and IFN- $\gamma$  by CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which was stronger in the presence of CT or two other immunostimulants—oleic acid or retinoic acid.

Together, these studies illustrate three important concepts of TCI: (1) Murine models are useful for evaluating the immunogenicity of transcutaneous antigen/adjuvant combinations. (2) Antigen-specific antibody and T cells can be generated in mucosal tissues of the oral cavity, large and small intestines, respiratory tract, and female reproductive tract using TCI. (3) As with other immunization routes, the magnitude and quality of the immune responses elicited by TCI is heavily influenced by adjuvant.

## 7 Mucosal Immune Responses After TCI in Humans

Obtaining approvals to evaluate new immunization strategies in humans is a difficult and lengthy process with ethical and safety considerations limiting the number of permutations that can be tested in individual studies. However, the striking advantages and safety profiles of TCI predicted from animal studies have paved the way to initiate a number of clinical trials. The safety of TCI in humans has been demonstrated in studies with live-attenuated virus (Etchart et al. 2007) and bacterial toxins (Glenn et al. 2000; Guarena-Burgueno et al. 2002; Frech et al. 2008). No severe adverse reactions were noted in three separate TCI studies. Vaccine recipients also rated the transcutaneous route as being more acceptable than the subcutaneous route (Etchart et al. 2007).

Etchart et al. (2007) performed TCI with a live-attenuated measles virus. Adults in this study had been immunized as infants with live-attenuated measles vaccine. While subcutaneous but not TCI enhanced serum IgG antibodies when compared to levels present before the trial, salivary IgA levels were elevated after transcutaneous but not subcutaneous immunization. TCI also induced virus-specific IFN- $\gamma$  production by peripheral blood mononuclear cells, suggesting a Th1-type response following the transcutaneous boost with measles vaccine.

TCI with *E. coli* LT, which simultaneously acts as antigen and adjuvant, induced LT-specific serum antibody that was enhanced by boosting at 12 weeks and further increased with a second boost at 35 weeks. Urine and fecal IgG and IgA were also detected (Glenn et al. 2000). When compared to placebo, the transcutaneous LT vaccine was effective for reducing the occurrence and severity of travelers' diarrhea caused by enterotoxigenic *E. coli*, indicating that TCI is safe and can impart protection against enteric disease (Frech et al. 2008).

In another study, adult volunteers received a dermal patch containing LT and the *E. coli* colonization factor CS6. The majority of vaccinees developed anti-CS6

serum IgG and IgA as well as delayed-type hypersensitivity responses. Modest immunogenicity was detected in people who received CS6 without the LT adjuvant (Guerena-Burgueno et al. 2002).

Transdermal vaccines against influenza virus infections would be very desirable, as these vaccines are intended for yearly mass administration to humans. Several studies have demonstrated the feasibility of TCI against influenza. Frech et al. (2005) demonstrated that rates of seroconversion against influenza antigens were increased after an LT-containing patch was applied on top of an injection site where a traditional influenza vaccine had been injected. This effect was particularly noticeable in elderly volunteers. A more recent study (Vogt et al. 2008) reported induction of effector CD4 and CD8 T cell responses as well as influenza-specific IFN- $\gamma$ -producing T cells after transcutaneous administration of a commercially available influenza vaccine. Although no adjuvant was given in this study, the skin was treated with superglue (cyanoacrylate glue) followed by tape stripping (Vogt et al. 2008).

Although safety and efficacy are still a concern for the development of transcutaneously delivered vaccines in humans, the studies presented here provide a solid foundation to support the advancement of promising TCI vaccines from the bench to the bedside.

## 8 Conclusion

The skin serves a critical protective role for the detection of and responding to invading pathogens. Immunization through cutaneous surfaces takes advantage of the assortment of immune-responsive cells in the skin to initiate an adaptive immune response. The past few decades have brought about a deeper understanding of the cutaneous cells involved in TCI and the important antibody and cell-mediated responses. Both human and murine studies support the use of TCI for induction of protective systemic and mucosal immune responses. There are many variables to consider with this route of immunization, such as site of administration, type of pretreatment if any, dosing, and the selection of appropriate adjuvant(s). Immune responses have been generated in multiple mucosal compartments (respiratory, digestive, and female genitourinary tract), making this non-invasive immunization route a promising vaccine delivery strategy for protection against a variety of pathogens.

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