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Review

Decision-making at the surface of the intact or barrier disrupted skin: potential applications for vaccination or therapy

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Abstract. The skin is a highly accessible organ and constitutes an active immunological site. Both these properties make this surface an attractive route for what promises to be a cost-effective, simple, practical and needle-free delivery of vaccines and immunomodulators. Less obvious is the fact that the state of the skin barrier can influence quantitative and qualitative aspects of antigen-specific immune responses. The everyday decision-making at the skin epithelium concerns the choice between the induction of an immune response and the establishment of a state of non-responsiveness (tolerance). This decision is influenced by various factors such as the dose, the route (intact vs barrier-disrupted skin), the cytokine microenvironment and the nature of the antigenic stimulus. By increasing our understanding of how immune responses are regulated in the epidermis we can envisage the development of immunisation protocols aimed at eliciting a protective immune response or inducing tolerance, with direct applications to preventive or therapeutic vaccination, respectively.

Key words. Skin immunity; skin barrier; vaccine; enterotoxin; adjuvant; autoimmunity.

Immunological features of the cutaneous microenvironment

The skin is one of the largest organs of the body representing an interface between the external environment and internal organs. With its complex structure and specialised derivative structures (various glands and hair follicles) it functions as a barrier. The stratum corneum, the outermost layer of the epidermis (10–25 μm thick in humans), provides the major skin barrier against water loss, and the various external physical, chemical and mechanical stimuli. This layer consists of dead keratinocytes (corneocytes) embedded in a lipophilic matrix. The epidermis is a stratified, continually renewing epithelium that exhibits progressive differentiation of keratinocytes in the basal to superficial direction. It is comprised of multiple cell types, among which the keratinocytes constitute 90–95% of the total epidermal cell population. These cells, together with the Langerhans cells (LCs), dermal dendritic cells, subsets of T lymphocytes that are effector cells in inflammatory reactions, and the strategically located lymph nodes, constitute the skin-associated lymphoid tissue [1, 2]. The dermal dendritic cells that are present in higher numbers than LCs represent a distinct differentiation pathway of dendritic cells with antigen-processing and -presentation capacity [3].

Keratinocytes play an active role in innate and adaptive immune responses by secreting cytokines, chemokines and anti-microbial peptides in response to various microbial pathogens and their components (fig. 1) [4–8]. These cytokines shape the local microenvironment and help to

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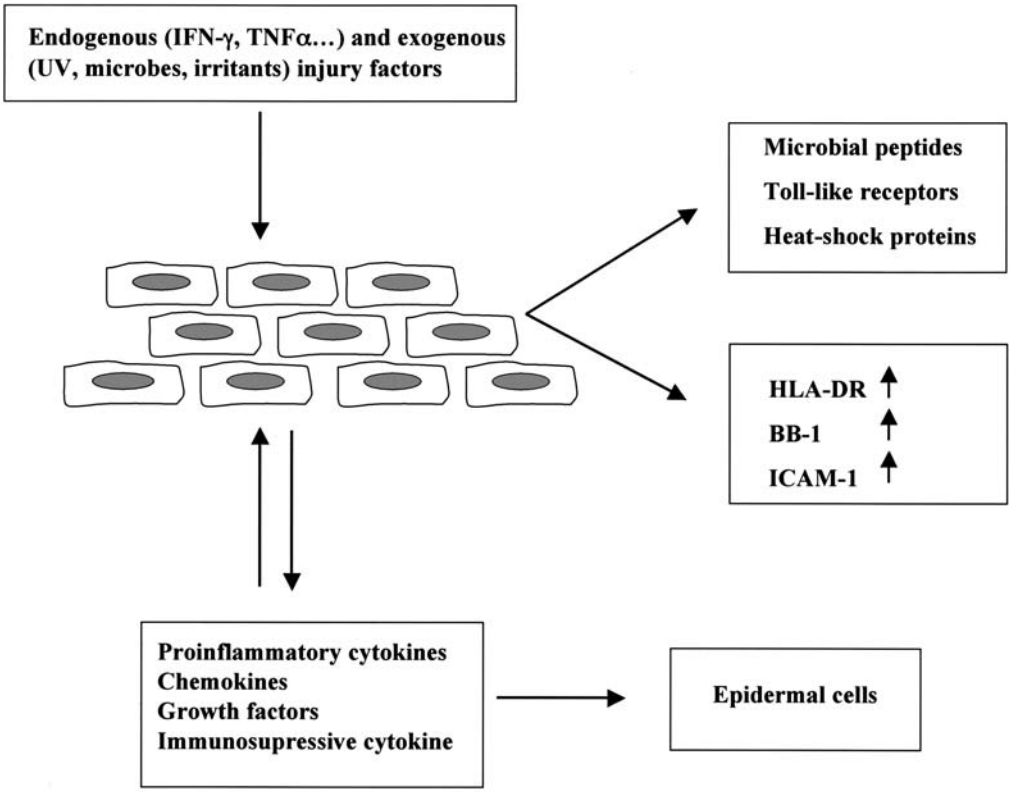

Figure 1. Role of keratinocytes in innate and adaptive immune responses.

maintain epidermal homeostasis [4]. Microbes and their components stimulate keratinocytes through pattern-recognition receptors, like the Toll-like receptors (TLRs) [9]. These receptors are part of the innate immune system, with TLR1, TLR2 and TLR5 being constitutively expressed by epidermal keratinocytes [10]. TLR4 is also present in the normal human epidermis and its expression is regulated by microbial components [11]. Keratinocytes also perform a scavenger function eliminating foreign material from the intercellular spaces of the epidermis [12]. Their role as antigen-presenting cells (APCs) is rather controversial, since they are generally deficient in expressing costimulatory molecules. However, keratinocytes have been shown to act as accessory cells for mitogen- or superantigen-driven responses [13].

LCs are dendritic epidermal cells that cover nearly 20% of the surface area through their horizontal orientation and long protrusions. They account for 2–8% of the total epidermal cell population and are defined by their dendritic morphology and the presence of a unique intracytoplasmic organelle, the Birbeck granule. They originate from bone marrow precursors, which, upon circulation in the peripheral blood, populate the skin where their life span is in the order of several weeks to several months. At the basal layer of the epidermis, immature LCs are sufficiently networked to exert their sentinel role by sampling and processing antigens. In the absence of a 'danger signal', LCs are present as a population of resting cells expressing low levels of cell surface major histocompatibility complex (MHC) class I and II molecules, and costimulatory or adhesion molecules [14]. This state of cells is particularly favoured by the local microenvironment created by the constitutive secretion of interleukin (IL)-10 and transforming growth factor (TGF)-β1 cytokines by the surrounding keratinocytes, as well as by nerve-derived peptides and various hormones [4]. Encounter with antigen in the epidermis or other stimuli (i.e. cytokines) favours the maturation and migration of LCs via the afferent lymphatics to the draining lymph nodes and gut mucosa where they present antigen to the resident lymphocytes [15–18]. With regard to their contribution to the induction of immune responses, LCs may produce antigen endogenously (i.e. after transfection following genetic immunisation) or take up exogenous antigen (i.e. diffused through the epidermis after topical application or produced by transfected cells, like keratinocytes). In the former case, processed antigens are presented by MHC class I molecules to CD8+ T cells, in the latter case, by MHC class II molecules to CD4+ T cells (fig. 2).

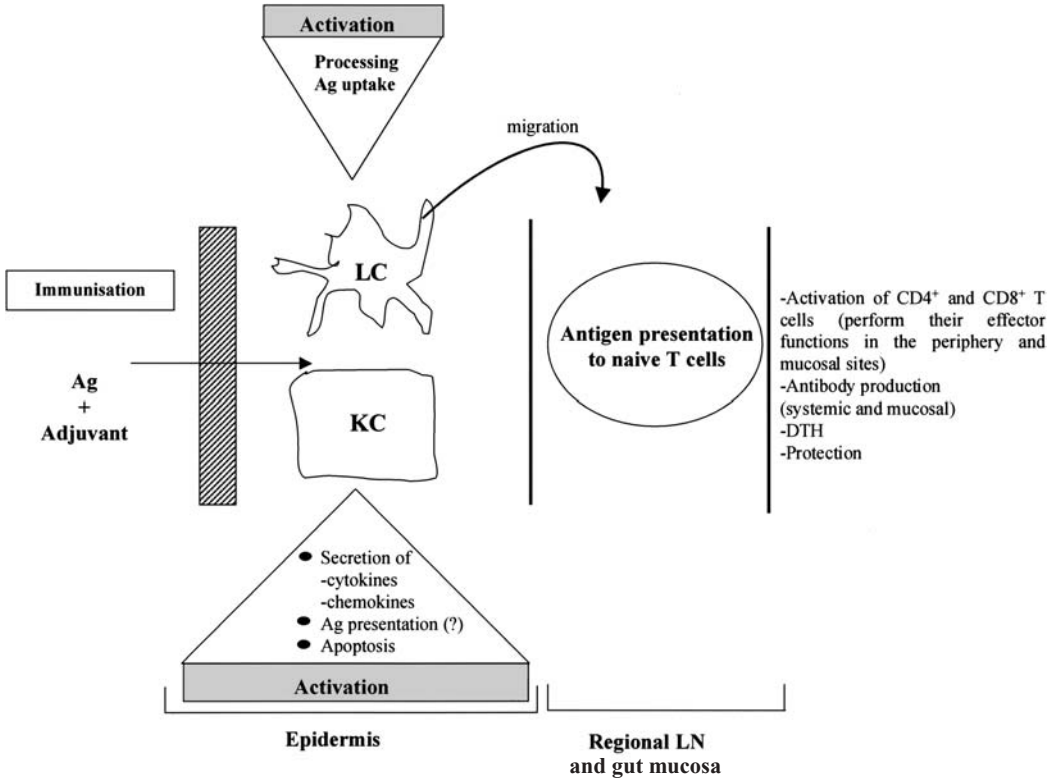

Figure 2. The role of LCs in the induction of immune responses following skin immunisation. Ag = antigen, KC = keratinocytes, LN = lymph nodes.

Disrupting the skin barrier for vaccine delivery and regulation of immune responses in the epidermis

The high accessibility of skin and the presence of immunocompetent cells in the epidermis makes this surface an attractive route for needle-free administration of vaccines [19, 20]. However, the lining of skin by the stratum corneum is a major obstacle to vaccine delivery. Advances in drug delivery have created new opportunities to successfully breach the skin barrier using devices that work with one or both of the following two methods: change in the physical environment of the skin and application of a driving force (table 1) [21]. A common characteristic of these methods is the disruption to various degrees (depending on the method) of the skin barrier, which results in the activation of LCs and keratinocytes. This ensures effective immune surveillance in the epidermis, repair of the barrier and establishment of epidermal homeostasis [4]. In particular, after barrier disruption, there is a chain of molecular events that leads to the secretion of proinflammatory cytokines like tumour necrosis factor (TNF)-α, IL-1α, IL-1β and granulocyte/macrophage-colony-stimulating factor (GM-CSF), by the keratinocytes [4, 22]. TNF-α and IL-1β facilitate the disassociation of LCs from keratinocytes by down-regulating the expression of E-cadherin, and the migration of LCs and/or their antigen presentation capacity by up-regulating the expression of a6 integrins, ICAM-1, CD86 and MHC class II molecules [23–25]. Moreover, they facilitate the movement of LCs from the skin by down-regulating the chemokine receptors CCR1/CCR5, and facilitating the homing of the LCs into the draining lymph nodes through the regulation of the CCR7 receptor [15, 26]. Interestingly, there seems to exist a functional heterogeneity within the epidermal LCs in response to signals that promote their migration, regardless of the nature of the stimulus. Only a proportion of LCs (approximately 30%) leave the epidermis. Some cytokines produced by keratinocytes orchestrate the metabolic responses required to repair the skin barrier. To this end, IL-6 and GM-CSF together with IL-1β and TNF-α contribute to the repair process [22, 27]. Disruption of the skin barrier also elicits the induction of anti-bacterial peptides that play a critical role in innate immune defences at the epithelial interface with the external environment against various pathogens [28]. Among these molecules, de-

Table 1. Delivering vaccines into the skin.

– Biolistic delivery using jet injectors
– Microneedle arrays
– Electroporation, microporation
– Use of particulate delivery systems
– Transcutaneous immunisation using ADP-ribosylating exotoxins as adjuvants and occlusive patch

fensins, which belong to a family of cationic anti-microbial proteins produced by neutrophils and macrophages have been shown to exert an adjuvant effect to coadministered antigens [29]. This suggests that they may play a role in regulating antigen-specific immune responses in the epidermis [30].

Of the cytokines produced by keratinocytes, IL-10 and IL-12 are critical for regulating cutaneous immunity favouring the induction of either Th1 or Th2 immune responses. IL-12 is known to promote Th1 immunity by stimulating the secretion of interferon (IFN)-γ, whereas IL-10 suppresses Th1 cytokine production by antigen-presenting cells (APCs) [31, 32]. Although keratinocytes constitutively produce low levels of IL-10, its expression in the epidermis is enhanced soon after barrier disruption [33]. Similarly, IL-4 expression in the epidermis is enhanced [34] and, therefore, these two cytokines contribute to preventing excessive inflammatory damage in the skin. In addition to cytokines, barrier disruption stimulates the secretion of chemokines, which are members of a family of small secreted proteins that modulate the migration of their target cells. In particular MIP-3a that is constitutively expressed in the skin is up-regulated in the epidermis after disruption of the skin barrier [35]. This chemokine is known to be a strong chemoattractant for LC precursors [36] required to warrant effective immunosurveillance in the epidermis.

Effect of skin barrier disruption on the immune responses to topically applied antigens

When an antigen is applied onto barrier-disrupted skin its percutaneous penetration is facilitated and, therefore, it is more readily available for sampling by the LCs. Moreover, disrupting the skin surface as described above is immunostimulatory, and this constitutes a signal that enhances and modulates antigen-specific immune responses [37, 38]. In the classical two-signal model for the induction of an immune response, signal 1 requires the recognition of antigenic peptides associated with MHC molecules by the receptors of naive T cells or the cell-membrane-bound immunoglobulins on B cells. Signal 2 is characteristic of professional APCs (expression of costimulatory molecules and secretion of cytokines) and is necessary to initiate primary T cell responses. In this view, the immunostimulatory effect that results from the impairment of the skin barrier is due to the attraction of LC precursors in the epidermis and/or activation and enhanced migration of antigen-bearing LCs from the epidermis to the draining lymph nodes. This process is promoted by chemokines or proinflammatory cytokines secreted from activated keratinocytes that favour or amplify a particular step in the cascade of the immunological events (as adjuvants do) leading to the induction of an immune response (signal 2). Accordingly, the level of barrier disruption will dictate the strength of signal 2 and the intensity of the immune response (fig. 3). Kahlon et al. [37] reported experiments demonstrating that coapplication of ovalbumin (OVA) and cholera toxin (CT) onto tape-stripped skin of mice enhanced the magnitude of anti-OVA antibody responses compared to those elicited after their application onto non-tape-stripped skin. Similar findings reviewed by Glenn et al. [38] using the cholera toxin B (CTB) subunit, tetanus toxoid or split-virus influenza vaccine as immunogens highlighted the importance of combining hydration and some disruption of the skin barrier to improve antigen delivery and en-

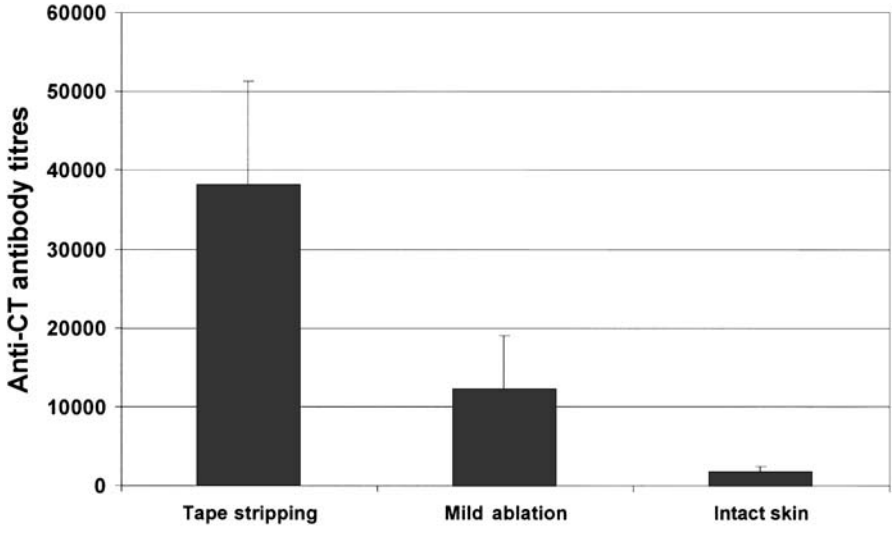

Figure 3. Antibody responses after a single application of cholera toxin (CT) onto intact or barrier-disrupted skin. Five BALB/c mice per group were immunised with 20 μg CT and antibody responses were measured by ELISA 2 weeks after topical application. Prior to topical application, the skin was hydrated for 5 min and then blotted with a dry tissue.

hance immune responses. Consistent with these observations are recent data showing that impairment of the skin barrier acts as an immunostimulatory signal sufficient to elicit robust cytotoxic T lymphocyte (CTL) responses after topical application of a tumour peptide antigen without an adjuvant [39]. Moreover, the primed tumour-specific CTLs protected mice against subsequent challenge with corresponding tumour cells, and suppressed the growth of established tumours [39]. In general, application of antigens onto barrier-disrupted skin without an adjuvant can elicit potent Th1- or Th2-type immune responses depending on the nature of the antigen and the degree of barrier disruption.

Decision-making at the surface of the intact skin and therapeutic implications

While the induction of immune responses against invading pathogens is important for protection of the host, immunity that is elicited against innocuous antigens, like allergens or self molecules can be harmful, leading to allergy and autoimmune diseases, respectively. Therefore, the everyday decision-making at the skin epithelium concerns the choice between the induction of an immune response and the establishment of a state of non-responsiveness (tolerance). This decision is influenced by various factors such as the dose, the route (intact vs barrier-disrupted skin), the cytokine microenvironment and the nature of the antigenic stimulus. Experiments in mice have demonstrated that repeated applications of an autoantigen, like the myelin oligodendrocyte glycoprotein peptide 35–55 with CT onto the intact skin of C57BL/6 mice, induced relapsing paralysis with demyelinating immunopathologic features similar to multiple sclerosis [40]. This is a chronic inflammatory disease of the central nervous system caused by infiltrating autoreactive CD4+ Th1 cells and activated macrophages. Similarly, the application of CT onto the intact skin of NOD/Lt mice with or without insulin B peptide 9–23 exacerbated insulitis [40]. Thus, contrary to the non-pathogenic Th2 responses elicited after systemic coadministration of CT with autoantigens [41], this enterotoxin is capable of promoting a different set of molecular events in the skin that contribute to processes leading to clinical disease. However, when myelin basic protein (MBP) was applied onto the intact skin of SJL/BL10.PL mice without an adjuvant, it induced tolerance [42]. The mice did not develop the relapsing-remitting form of experimental allergic encephalomyelitis (EAE) when they were subsequently injected with MBP in a complete Freund's adjuvant emulsion, intradermally [42]. More recently, protection against the spontaneous or induced forms of EAE was demonstrated with the immunodominant peptide Ac1-11 from MBP when it was applied without adjuvant onto the intact skin of mice bearing a transgene for the Ac1-11 T cell receptor [43]. This suppression was antigen specific and dose dependent and was mediated by CD4+/CD25– T cells [43].

In addition to autoantigens, protein molecules or haptens when applied with a patch onto the intact skin of mice in the absence of adjuvant suppressed the Th1-mediated delayed hypersensitivity reactions to the homologous antigen, sensitised the animals, and elicited a predominant Th2-type response characterised by the production of high levels of antigen-specific IgE antibodies [44–46]. This state of Th2 activation that characterises atopic individuals was shown to be IL-4 independent, contrary to what is normally observed in Th2-type responses elicited after mucosal exposure [47]. Thus, the skin, like mucosal surfaces being constantly exposed to allergens, is a particularly potent site for Th2 sensitisation. Biasing the cytokine profile to the Th1-type may, therefore, have potential therapeutic applications. Support for this notion comes from experiments demonstrating that CTB could potentiate antigen-specific Th1-driven responses and significantly reduce allergen-specific IgE antibodies in sensitised mice after transcutaneous immunisation [48]. CTB is composed of five B subunits and is responsible for the binding of CT to GM1 ganglioside receptors present on all nucleated cells [49]. What is remarkable with this molecule is its suppression of Th1-associated immune responses when it is administered via the mucosal routes [50]. This underlines the importance of the local microenvironment for the expression of different sets of properties for a given molecule. Another strategy to modulate the nature of the immune response is to use CpG motifs. These are unmethylated sequences derived from bacterial DNA that directly activate APCs to release high concentrations of Th1 cytokines and express increased levels of cell surface costimulatory molecules [51]. Given their potent immunostimulatory properties, synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (ODN CpGs) have been considered candidate adjuvants for vaccines or immunomodulators for therapeutic applications [52–53]. After deposition onto bare skin they have been shown to exert an immunomodulatory effect shifting immune responses to Th1 and acting synergistically with CT [54, 55]. Their effect was more pronounced when lower doses of CT were used in the CT/ODN CpG mixture [55], which could be advantageous given the fact that high doses of CT can favour the induction of autoimmune reactions [40]. In addition to CT, a critical question concerns the nature of oligonucleotides that are used in these preparations, since, for example, pI:pC motifs known to mimic viral dsRNA have been shown to activate immature dendritic cells to a stage where they act as professional APCs and can be arthritogenic [56].

Overall, these findings highlight the potential risks of bacterial enterotoxins with ADP-ribosylating activity in

favouring the development of an autoimmune disease in a genetically susceptible individual but also the advantages of considering the intact skin as a surface to induce suppression or immunomodulation for prevention or therapy of chronic inflammatory autoimmune diseases or certain allergic conditions, respectively.

Perspectives

Vaccine delivery via the skin has drawn a lot of interest mainly due to its potential advantage in overcoming the requirement of needles and syringes. So far, research has been largely empiric, testing various types of antigen, adjuvant, vaccination schedule and delivery approach. This has helped to establish some basic principles of vaccine delivery and provided a better understanding of the complex mechanisms involved in the regulation of skin immunity. However, our knowledge of the mechanisms by which enterotoxins that are used as adjuvants in skin immunisation protocols regulate immune responses in the skin is clearly incomplete. A key question is how topical application of CT onto bare skin can trigger the activation and/or expansion of autoreactive T cells that are able to elicit inflammation at distant sites. A large number of autoimmune diseases are characterised by skin alterations. Studying the immunological effects of CT or mutants of enterotoxins with lower ADP-ribosylating activities may provide useful information relevant to physio-pathological alterations of the immune system linked to skin in these diseases.

The advent of new delivery technologies together with current progress in producing more refined antigen preparations and elucidating key mechanisms of adjuvant activity will certainly give new impetus to the development of vaccines that can be administered in a simple, practical and painless way via the skin.

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