

Skin as a novel route for allergen-specific immunotherapy

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Running title: Immunotherapy via skin

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Abstract

Subcutaneous allergen specific immunotherapy is an effective treatment of IgE-mediated allergies, but it requires repeated allergen injections with a risk of systemic allergic reactions. Other routes of immunization had been explored to improve patient compliance and safety. Skin is not only a physical barrier between the body and outside world, but also an important immune organ eliciting innate and adaptive immune function. Skin has been used as an ideal site for vaccination of infectious diseases. Food allergen topically applied onto disrupted skin can induce sensitization and food allergy would develop subsequently. However, immune tolerance would be induced if the skin barrier is kept intact. Several mice and human studies on epicutaneous immunotherapy showed successful treatment on IgE-induced allergy models or allergic diseases. Migratory Langerhans cells might play a decisive role in the induction of different immune responses. Further research on the underlying mechanism of the crosstalk between skin and gut or airway is helpful for the understanding of many protective or sensitizing immune responses induced via skin, and also is helpful for the development of new strategy for the treatment of allergic disease.

Keywords: skin; food allergy; immunization; tolerance; immunotherapy; epicutaneous; Langerhans cells; peanut.

Introduction

It is now appreciated that anaphylactic allergy to foods is far more common than was realized only a decade ago, and is occurring with an increasing prevalence/incidence [1-3]. Food allergy affects up to ~3.7% of the overall US population and 6% to 8% of young children [4]. Peanut allergy, which affects around 1% of the population, is among the most severe food allergies and causes life-threatening clinical reactions including death. Among IgE mediated food allergies, peanut, tree nut, egg and milk allergies are the most prominent, especially in children, of which peanut allergy is involved in most deaths [5]. In contrast to the expanding array of treatments available for inhalant and cutaneous allergic disorders, currently, the only available treatment for allergic reactivity when accidental peanut ingestion occurs is rapid self-administration of injectable epinephrine. Strict avoidance to prevent reactions is not only nearly impossible, but also a major lifestyle intrusion for both the patient and his/her family.

Allergic responses are strongly associated with Th2 type skewed immune responses and antigen-specific IgE production. Modulation of the skewed Th2 response toward a more balanced response is the major goal of allergen immunotherapy (IT) for allergic disorders. Such therapy can be curative in that once given for a period of years, treatment can be discontinued without return of symptoms, e.g., “allergic tolerance” has been achieved. To this end, protein-based allergen IT with subcutaneous (s.c.) injection has been widely used in clinical practice. However, the efficacy of such allergen IT is variable, a long duration (several years) of frequent treatments is required, and the conventional allergen IT *via* s.c. injection of the escalated dose of the corresponding allergens can unpredictably trigger local and systemic allergic responses. Of particular

relevance to food allergies is that the conventional protein-based IT has proven repeatedly to be too dangerous to undertake in those with severe (anaphylactic) sensitivity, as fatal reactions to IT have occurred even under investigational conditions. Therefore, other approaches, such as anti-IgE therapy [6], traditional and modified peptide immunotherapy [7, 8], oral immunotherapy [9], mutated allergen proteins [10-12] and homologous proteins [13]; allergen immunotherapy plus Th1-response enhancing adjuvants, e.g. CpG [14]; traditional Chinese medicine [15, 16]; and allergen gene vaccinations [17] are all being examined to try to overcome the problems accompanying allergen IT in severe food allergic patients. However, these approaches have yet to yield satisfactory results, and the current therapeutic pipeline in this area is not robust. Anti-IgE may provide some level of ongoing protection for severe food allergy, but it certainly is not the long term “answer”, as passive anti-IgE immunotherapy has shown only limited success in reducing allergic reactivity and would require uninterrupted life-long treatment. Studies are now examining whether prevention of the development of severe food allergy can be achieved in high-risk subjects (ITN website) by early life high dose exposure but this approach will not address the needs of those who are already severely allergic to foods. For these reasons, it is universally agreed that novel approaches for the treatment of established severe food allergy are urgently needed.

1. Skin immunization as an efficient vaccination strategy

Stimulating the immune system for “optimal” immunization/vaccination outcomes is the major challenge in vaccine development, and the “optimal” outcome in one setting may be quite different than that in another, *e.g.* protection against influenza vs. protection

against allergy. Differing vaccination approaches have been extensively investigated in order to replace the traditional intramuscular vaccination route now recognized as suboptimal for vaccination, due to the limited availability of functionally active antigen-presenting cells (APCs) and the lack of expression of MHC-class II and costimulatory molecules in myocytes that renders them incapable of directly priming T cells. The outermost APCs of the epidermis, Langerhans cells (LCs), cover about 20% of the skin surface area. They initially contact and capture the topically applied antigen, and therefore play a key role in controlling cutaneous derived immune responses. The strategic location and arrangement of the immunocompetent cells in the skin, particularly the LCs in the epidermis and different dendritic cell (DC) subsets in the dermis, form a powerful pleomorphic immune network called the skin-associated lymphoid tissue (SALT) [18]. Although the cutaneous route has long been recognized as an attractive target for vaccine administration, it has been under-explored in terms of modulating systemic and/or gastrointestinal immune responses. Another attractive feature of cutaneous immunization is its easy and needle-free methodology.

Increased understanding of immunology of the skin has shown that immunization *via* the skin may be a valuable strategy for both preventive and therapeutic vaccines against infection and tumor [19, 20]. Murine and human studies have demonstrated that delivery of antigens via skin can be an effective method of inducing immune responses, including humoral and cellular immune responses to protein and peptide antigens, generally in the presence of an adjuvant such as cholera toxin (CT), heat-labile enterotoxin or their detoxified mutants, or immunostimulatory CpG [21-24]. A growing body of evidence suggests that this route may be particularly beneficial to certain higher-risk populations,

such as the elderly, the immunocompromised and cancer patients, e.g. improved antibody responses could be obtained in elderly individuals vaccinated against influenza *via* skin immunization [25].

The integrity of the skin and the use of adjuvants, along with the nature of the antigen, its dose and timing of inoculation(s), are among the important factors in determining the nature of immune responses induced via cutaneous immunization [18]. Normal skin is covered with a layer of the stratum corneum, which is a barrier that limits the efficient penetration of the topically applied Ag for reaching the skin APC (LCs and/or dermal DC subsets). Disruption of this skin barrier, such as repeated tape stripping or the use of DMSO, has been used to enhance the ability of antigen to cross this barrier and induce enhanced immune responses [26]. Importantly, these procedures that disrupt the skin barrier not only increase the Ag penetration, but also function as stimulatory process that result in the activation of local LCs and keratinocytes that, through multiple mechanisms, result in enhanced and often Th1 or Th2 deviated immune responses. In particular, a chain of molecular events leads to the localized secretion of proinflammatory cytokines such as TNF- α , IL-1 α , IL-1 β and GM-CSF by the keratinocytes and the recruited inflammatory dendritic epidermal cells [27, 28]. These microenvironment changes facilitate the disassociation of LCs from keratinocytes by down-regulating the expression of E-cadherin, modifying the migration of LCs and/or changing their antigen presentation capacity by up-regulating their expression of α 6 integrins, ICAM-1, CD86 and MHC class II molecules [29-31]. Moreover, these changes 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 [32]. While important in driving enhanced immune responses via the skin, it is our contention that such changes are antithetical to induction of allergic tolerance.

2. Skin as a potent sensitization route inducing allergy

Although oral route is likely the major route for peanut allergy sensitization, other routes, such as inhalation and/or skin contact with peanut protein products also have been proposed as routes for peanut sensitization, as many peanut allergic children have no identifiable oral peanut consumption that apparently associated with the peanut sensitization [1, 2]. However, it is just as likely that unappreciated low dosage oral exposures, in particular to peanut, are what drive the initial sensitization. In one retrospectively epidemiological analysis, an association between topical use of skin care products containing peanut oil and the development of peanut allergy was reported [33, 34]. However, such observation has not been further verified and no reliable data are available regarding epidermal dose of peanut allergen required to induce sensitization. Thus, induction of sensitization against peanut proteins through contact with the skin via skin care products and the respective protein concentrations remains a matter of speculation. Additionally, applications of skin care products, when given for atopic dermatitis, are being presented via highly inflamed skin in the context of a strong Th2 deviation. Animal experiments have shown that contact sensitivity and allergic dermatitis-like appearance can be modeled with repeated topical contact with experimental allergen such as ovalbumin through gauze skin patch and repeating tape stripping that disrupt the normal skin barrier function [35-38]. Oral tolerance could be prevented and allergic sensitization enhanced when peanut proteins were applied to the

skin via the structurally disrupted skin [39].

It appears that in all these observations, the structural disruption of the normal skin through patching or tape stripping was a prerequisite, as experimental allergic sensitivity could not be produced via normal undisrupted skin. This appears to be because under normal conditions, LCs constantly monitor the epidermal compartment for foreign antigens/pathogens, inhibit T cell activation, and maintain a state of unresponsiveness or tolerance to the environmental antigens they are constantly exposed to. In contrast, in diseased skin such as atopic dermatitis, the proinflammatory cells such as inflammatory dendritic epidermal cells (IDECs) are recruited into the epidermis [26]. In response to allergens, pathogens and local inflammation/stimulation, IDECs produce proinflammatory mediators that display strong stimulatory activities toward T cells, leading to the exacerbation of the allergic inflammation in the skin [40]. Additionally, the cytokine thymic stromal lymphopoietin (TSLP) would be produced by keratinocyte after the application of vitamin D3, which results in an atopic dermatitis-like syndrome [41]. Such a scenario probably accounts for the significantly different response to the antigen deposition to the structurally intact and diseased skin.

3. Skin as a route inducing immune tolerance.

The Th2-type immune responses described above that were induced by application with antigen by patching on intact skin might suppress Th1-mediated responses causing peripheral tolerance. In the past several years, Szczepanik's group explored the effect of skin application with antigen on intact skin by patching, on several disease models characterized by Th1-mediated immune response. Their data showed that cutaneous

immunization of mice with different protein antigens applied on the intact (without tape-stripping) skin in the form of a patch or cream emulsion induced a state of subsequent unresponsiveness upon antigen challenge [42, 43], which they termed “skin-induced tolerance”. Such skin-induced immune tolerance has been demonstrated in several experimental systems, including experimental autoimmune encephalomyelitis (EAE) [44, 45], contact sensitivity [46], collagen-induced arthritis [42], organ transplantation rejection [47], and TNBS-induced colitis [48]. More recently, it was found that besides the Th1-mediated contact hypersensitivity, epicutaneous immunization with hapten-conjugated protein antigen induces Treg cells that inhibit CD8 Tc1-mediated contact sensitivity in mice [49,50]. These observations, along with the fact that robust immune responses can be induced via skin-immunization of various antigens in the presence of adjuvant, suggest that appropriate manipulation of the SALT can be used to provide an effective means to regulate immune responses either positively or negatively.

Mechanistic studies of skin-immunization induced immune tolerance showed that this type of tolerance is mainly due to the activation/induction of the negative regulatory T cells (Ts) that inhibit sensitization and elicitation of effector T cell responses [42]. Inhibition was transferable *in vivo* by TCR $\alpha\beta$ +CD4+CD8+double positive lymphocytes harvested from lymphoid organs of skin-patched animals, and was mediated via TGF- β [42, 51]. Such skin immunization induced tolerance for contact sensitivity could be reversed with the TLR4 ligand LPS that activates contra-suppressor T cells, which belong to a population of TCR $\beta\alpha\beta$ +CD4+lymphocytes, to reverse the tolerance status with antigen-specific manner. Induction of these contra-suppressor T cells was MyD88 dependent and the reversal of tolerance (or suppression) was dependent on IFN- γ and

IL-12, but not IL-6 [52]. The LPS-mediated reversion effect was not observed in the *Tlr-4* mutant C3H/HeJ mouse, indicating that this effect was dependent upon the intact TLR-4 signaling [53]. Although it's well accepted that contact sensitivity is mediated by T cells, more recently it was shown by Paust et al that contact sensitivity can be also mediated by liver NK cells [54]. And this type of contact sensitivity can be suppressed by epicutaneous immunization with DNP-BSA [55].

In a more recent study, the Szczepanik's group conducted a double-blind, placebo-controlled, clinical study in multiple sclerosis (MS) patients to assess immune effects of a mixture of 3 myelin peptides applied in an adhesive patch to the intact skin [56]. The data showed that myelin peptides was feasible and tolerogenic in MS patients. The myelin peptides that applied epicutaneously to MS patients, activated dendritic Langerhans cells in the skin and induced a unique subpopulation of DCs in lymph nodes draining the immunized area of the skin. In the periphery, epicutaneous immunization with myelin peptides induced type 1, IL-10-producing Tregs and strongly suppressed myelinreactive T-cell responses. Thus, their results demonstrated for the first time an immunoregulatory potential of epicutaneous immunization with myelin peptides in MS patients [56].

For allergy tolerance, our group reported that topical application with peanut allergen on intact skin inhibited subsequent peanut sensitization. In an effort to establish an adjuvant-free peanut allergy sensitization mouse model via cutaneous sensitization, we observed that the cutaneous administration of a range of relatively high doses of crude peanut extracts to the undamaged skin, rather than sensitizing the animals, rendered them resistant to the induction of peanut specific IgE upon subsequent oral peanut sensitization

[57]. This suggested that allergy tolerance to oral peanut was the result of the high dose skin immunization. To show that this was a generalized phenomenon, skin allergen application-induced oral tolerance was also reproduced using OVA as an experimental allergen. Thus, our results drive us to test the hypothesis that cutaneous induction of tolerance to severe food allergy may be a novel therapeutic option for the prevention/treatment of IgE-mediated food allergies. Developing the skin-immunization based peanut allergy tolerance as a goal for therapeutic immunotherapy would be particularly practical and realistic for peanut allergy and/or other severe food allergies where preventing the development of or decreasing the clinical severity of the reactions is a high priority. Cutaneous induction of food allergen tolerance should have the advantage of improved safety and ease of administration as compared to the current trial of oral administration.

The key point of our protocol is that we applied antigen directly onto the shaved skin without patching, thus our treatment doesn't induce Th2-type immune response characterized by production of IgE and IgG1. All of the studies utilizing a patch would elicit a Th2-type immune response, as shown in Szczepanik's group's work and many other studies [58-62]. Covering of patch on skin would change the status of skin barrier by producing more moisture, and physically stimulate the keratinocytes and Langerhans cells in the epidermis, and facilitate the growth of skin microbiome. All of these changes would have effects on the immune reaction induced by the antigens applied via the patch. Thus under the patch the skin is no more "intact", but "disrupted". And skin application of antigens/allergens to "disrupted skin" can induce local dermatitis-like inflammation and promote immunization, allergic sensitization and even systemic immune tolerance

[58-62]. For induction of allergic sensitization, structural disruption of the normal skin via various approaches, such as repeated patching or tape stripping, was a prerequisite as sensitization was not achieved with undisrupted skin [58-62]. Our results showing that the defined exposure of food allergens to intact skin suppresses the subsequent food allergy oral sensitization, suggesting that such an approach has the potential as an alternate for peanut and/or other food allergy tolerance induction.

Application of antigen on intact skin in our study would induce the production of regulatory immune cells and cytokines, although the Th2-type immune response can't be induced. Our results showed that biopsies from the allergen skin application sites did not display any evidence of inflammatory changes as expected. In contrast, the cytokine expression profiles and the increased Treg and migratory Langerhans cells from the local draining ILNs showed that local DC- and T-cell mediated responses were indeed induced by the topical allergen application. These local effects are likely the result of cutaneous dendritic cells, Langerhans cells migrating to the local lymph nodes. The increased IL-2, IL-10, and IFN- γ and lack of IL-4 induced by the higher CPE dose suggests that the high dose cutaneous CPE application is capable of promoting a local regulatory T cell response [57].

4. Epicutaneous allergen administration as a novel method of allergen-specific immunotherapy

As skin showed the capacity of inducing immune tolerance, many investigators are exploring the possibility of using skin as a novel route for allergy-specific immunotherapy. Actually the clinical efficacy of epicutaneous allergen administration

was reported more than 50 years ago, when a French allergy specialist performed allergen-specific immunotherapy using needle scarification of the volar forearm and described improved or complete relief from hay fever (for as long as 3 weeks) in most of the treated patients [63]. More recently Senti et al reported the results from a phase I/II study to evaluate the safety and efficacy of epicutaneous administration of specific allergen immunotherapy by using grass pollen allergens in patients with seasonal rhinitis [64]. The authors applied pouches filled with petroleum jelly that contained *Phleum pratense* extract to the patients' upper arm for 48 hours, once weekly for 12 weeks, after tape-stripping of the skin. They report that, during the pollen season, patients given epicutaneous administration of grass pollen allergens had significantly reduced symptoms compared with patients given placebo. It is worthy of noting that tape-stripping is prerequisite for their protocol. Tape stripping of the skin induces a bystander effect mediated by keratinocyte-derived proinflammatory cytokines, an effect that cannot readily be achieved in sublingual immunotherapy. The strength and efficacy of antigen presentation and lymphocyte stimulation strongly depend on these bystander reactions, inducing secretion of IL-1, IL-6, IL-8, and TNF- α [65-68]. It is also reported that tape stripping can induce production of IL-12 and IFN- γ in the skin [69] and increase expression of MHC class II, CD86, CD40, CD54, and CD11c on Langerhans cells [70], as well as enhanced expression of Toll-like receptor 9 in keratinocytes [71], producing an environment favoring induction of allergy-protective immune responses mediated by Langerhans cells [65, 72]. However, the possibility does exist that tape stripping could also induce skin-sensitization under proper co-stimulating conditions, and this might account for the moderate effect of the EPIT for the treatment of allergy [64]. In a more

recent report, Senti et al used a 95mm X 95mm patch containing different doses of grass pollen extract for the EPIT, and got similar results as before, which further confirmed the efficacy and safety of EPIT [73]. They also tested the adjuvant potential of diphenylcyclopropanone, a strong contact sensitizer, to facilitate the penetration of protein through the stratum corneum in EPIT. This preclinical study showed that diphenylcyclopropanone enhanced antigen-specific IgG2a antibody responses as well as IL-10 cytokine production after epicutaneous immunization with OVA. EPIT with OVA and DCP also protected sensitized mice from anaphylaxis and asthma, which was more robust than that of conventional SCIT [74].

By applying a device delivering allergen onto intact skin, a French group showed exciting results for epicutaneous immunotherapy (EPIT). They reported encouraging results of epicutaneous immunotherapy for children allergic to cow's milk [75] and published EPIT preclinical data from mice using Viaskin (DBV Technologies, Paris, France), a new epicutaneous delivery system (EDS) that allows application of various allergens to the skin [76, 77]. Four allergens were used in their study: pollen, house dust mite, ovalbumin and peanut. Mice were sensitized by subcutaneous injection or intra-gastric gavages for sensitization. Then the mice were treated with a device called Viaskin, a new patented EDS developed by DBV Technologies. Viaskin is composed of a central translucent polyethylene membrane (11mm in diameter) surrounded by an adhesive polyester non-woven crown as contact support to maintain the chamber on the skin. A dry layer of allergenic materials, containing OVA, pollen, HDM or peanut proteins was deposited on this backing (100 µg total protein of each allergen). The delivery system creates an occlusive chamber on the skin that rapidly generates moisture

and releases the allergens from the plastic support to the skin, allowing adequate diffusion of the proteins towards the epidermal immune-competent cells. After the hair on the back of mice were removed by depilatory cream, mice were anaesthetized and EDS, with 100 mg OVA, pollen, HDM or peanut proteins, were placed on the back of the mice and maintained using a bandage for 48 h. The EPIT group showed decreased level of allergic response and lower level of allergen-specific IgE. This pilot study suggests that immunotherapy can be achieved with repeated and prolonged applications of allergens to the intact skin. This so-called EPIT shows an efficacy profile comparable to SCIT, considered in this study and in clinical practice as the reference method.

When applied onto intact skin, the EDS preserves its architecture, including the more superficial layers. Experiments with labeled Ags in *in vitro* transdermal models have shown that this EDS allows native protein to concentrate inside the stratum corneum within the vicinity of immunological cells, without passage through the skin [78]. In the ensuing study for the mechanism, they demonstrated that epicutaneous application of an allergen onto intact skin leads to its transport via DCs to the draining LNs, with variations according to the previous immunological status toward this allergen and the timing of the application [79]. A prolonged and repeated application of an allergen by EDS onto intact skin of sensitized animals induced a down-modulation of allergen-specific local and systemic responses possibly through the induction of Tregs [79].

5. Skin-mucosa interactions

As the two largest immune organs in the body, the skin and the gut mucosa are not only the main physical barriers for external pathogens, but also serve as active sites for

inducing the immune responses as well as maintaining immune tolerance. The skin and mucosa share many structural and functional similarities so that the skin is generally considered to be a “distant relative” of a mucosal surface. It is well known that immunization with an antigen via the gastrointestinal tract or nasal mucosa may lead to both a local immune response and a state of profound immunosuppression in the periphery [80-82]. This peripheral suppression plays an important role in avoiding the development of immune responses to non-pathogenic antigens such as food, albeit the detailed mechanism(s) for this compartmentalization of immune responses/tolerance remains unknown. It has been suggested that certain types of regulatory T cells are preferentially induced in mucosa to maintain tolerance [83, 84]. Similar to the mucosa immunity, cutaneous immunization can generate multiple types of immune responses in the presence of appropriate adjuvant, including the robust antibody responses (typically IgA and IgG) and cell-mediated immune responses (CD8-mediated T cell cytotoxicity) in the mucosa of the gut, lung, mouth and female reproductive tract [23]. At the same time, as discussed in section 3 (Skin-immunization induced immune tolerance), cutaneous immunization can induce the peripheral immune tolerance. Thus, the skin based immune system (e.g., SALT) and the mucosa-based immune system (e.g., GALT) communicate and interact with each other to maintain the overall homeostasis of the immune responses *in vivo*.

The mechanism by which skin-immunization induces immune responses in both systemic and multiple mucosal compartments is largely unknown in the context of food allergy. A particularly interesting and important aspect of the skin-mucosa interaction is how the modulation of the immune response in the gastrointestinal mucosa is achieved

via skin immunization. The current data suggest that the migration of the antigen-loaded DCs from skin to both systemic and mucosal compartments to activate the resident T cell *in situ*, and the homing of the $\alpha 4\beta 7^{+}$ T cell to gut mucosa might be the responsible mechanisms for the skin-immunization induced gut mucosa immunity [85, 86].

In summary, skin has been recognized as an important immune organ with the progress in the function of Langerhans cells, keratinocytes and regulatory cells in immune regulation. The integrity of the skin and the use of adjuvants, along with the nature of the antigen, its dose and timing of inoculation(s), are among the important factors in determining the nature of immune responses induced via cutaneous immunization, sensitization or tolerance. Proper manipulation of allergen onto skin would be a promising method for future treatment of allergic disease.

Abbreviations:

APCs: antigen-presenting cells;

IT: immunotherapy;

LCs: Langerhans cells;

DC: dendritic cell;

SALT: skin-associated lymphoid tissue;

CT: cholera toxin;

IDECs: inflammatory dendritic epidermal cells;

EPIT: epicutaneous immunotherapy;

EAE: experimental autoimmune encephalomyelitis;

EDS: epicutaneous delivery system.

MS: multiple sclerosis

TSLP: thymic stromal lymphopoietin

Conflict of interest: The authors declare no conflict of interest.

Acknowledgement:

We thank Andrew SAXon for his helpful discussion and critical reading of the manuscript and language correction. This work was supported by grants from the National Natural Science Foundation of China (30972641, 31070793, 81273320, 81271750).

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