

# Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response

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The skin is an important immunological organ with an outer protective layer, the stratum corneum forming a barrier between the skin-associated lymphoid tissue and the environment. We show that gently removing the stratum corneum with adhesive tape permits potent epicutaneous immunization to protein antigens. IL-4 secretion by T cells from draining lymph nodes and high levels of specific IgE and IgG1 with no IgG2a showed that the immune responses induced following epicutaneous antigen exposure are strongly Th2 biased. Similar responses were obtained with different antigens and mouse strains. In contrast, subcutaneous immunization with antigen delivery into the dermis was less potent and gave predominantly Th1 responses. Removal of the stratum corneum increased expression of MHC class II, CD86, CD40, CD54 and CD11c on Langerhans cells, but did not cause them to migrate. Rapid migration from epidermis to draining lymph node was obtained, however, by exposure to antigen after removal of the stratum corneum, suggesting that maturation and migration of Langerhans cells are independently regulated events. These results suggest that antigen presentation by Langerhans cells gives predominantly Th2 responses. This may provide an explanation for allergic sensitization to some antigens. It may also be a useful non-invasive, non-adjuvant-dependent method of vaccination.

**Key words:** Skin / Th1/Th2 cells / Allergy / Langerhans cells / Vaccination

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## 1 Introduction

The skin is naturally exposed to a variety of environmental agents including allergens. The stratum corneum is a protective horny layer of keratinin-filled dead and dying cells on the skin surface that acts as a barrier against microbes and environmental antigens [1]. Beneath the stratum corneum is an epithelial layer of keratinocytes separated from the dermis by a thin basement membrane. Interspersed among the keratinocytes are specialized antigen-presenting Langerhans cells (LC). These are members of the wider family of dendritic cells (DC) but are phenotypically and functionally distinct from dermal DC that reside in the dermis [2]. LC form a contiguous network in the epidermis where they serve as senti-

nels of the immune system by sampling antigens that get through the stratum corneum and responding to signals, such as inflammatory cytokines, produced by the surrounding keratinocytes. They can then relay antigenic information to the adaptive immune system by migrating from the epidermis to draining lymph nodes (LN) [3, 4].

In inflammatory diseases of the skin such as atopic dermatitis, the barrier properties of the stratum corneum are diminished and otherwise harmless environmental agents may cause irritation and inflammation. Extracting lipids from the stratum corneum with organic solvents or removal of the stratum corneum by mechanical tape stripping initiates a chain of biological events in the epidermis very similar to that seen in inflammatory skin diseases. The proinflammatory cytokines TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and GM-CSF are rapidly produced [5–7] and expression of MHC class II and costimulatory molecules, such as CD86 and CD54, on LC is increased [6]. TNF- $\alpha$ , IL-1 $\beta$  and IL-18 all play important roles in the induction of LC migration from the epidermis [8–10], and may also promote the maturation of LC into immunostimulatory DC.

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**Abbreviations:** LC: Langerhans cell DTH: Delayed type hypersensitivity CLN: Cervical lymph node MLN: Mesenteric lymph node

Such augmentation of immune reactivity may be critical for the elimination of microbes or other environmental antigens that gain access to the barrier-disrupted epidermis, but it may also result in unwanted immune and/or inflammatory responses to innocuous agents and antigens. Exposure of inflamed skin to allergens may result in some forms of atopic dermatitis and/or systemic allergic diseases. Food allergy and skin diseases such as atopic dermatitis are interlinked, although the mechanisms involved remain unknown [11]. Most food allergic children also suffer from atopic dermatitis, which usually predates development of the food allergy [12, 13]. Of individuals with a peanut allergy, 95% suffer from other atopic diseases, and atopic dermatitis is particularly prevalent. Approximately 80% react to their first known oral exposure to peanut, implying some prior occult sensitization of which the skin is a likely possibility [14]. Primary sensitization through the skin could occur from the use of topically applied creams and lotions, where peanut oil has been a common ingredient, to manage atopic dermatitis.

Protein allergens can elicit eczematous inflammatory reactions in the skin as in the atopy patch reaction [15], and it is becoming clear that exposure of skin to protein antigens can also stimulate a primary and systemic immune response. Prolonged skin exposure to ovalbumin (OVA) in an occlusive patch applied to shaved skin induced typical Th2 responses in mice, and animals showed systemic allergic sensitization and hyper-responsiveness to methacholine after inhalation of OVA [16, 17]. The chemical hapten picryl chloride applied to shaved and barrier-disrupted skin has also been shown to elicit a Th2-dominant response that is clearly distinct from the response induced through intact skin [18].

In this study, we report that aqueous solutions of common food protein antigens (peanut protein and OVA), when applied to the epidermal surface after removal of the stratum corneum with adhesive tape, induce primary antigen-specific and systemic immune responses that are strongly Th2 biased. In contrast, injection of antigen into the dermis gives rise to Th1-type responses. Our results suggest that LC in the epidermis play a pivotal role in promoting Th2 responses and propose that exposure to common antigens on disrupted skin surfaces may partly explain the increase in allergic responses to environmental allergens in the developed world.

## 2 Results

### 2.1 Removal of the stratum corneum allows primary systemic epicutaneous immunization to protein antigens

Histological examination of hematoxylin-eosin-stained sections showed that five to eight repetitions of adhesive tape stripping completely removed the stratum corneum from the skin of the ear without disrupting the integrity of the underlying epidermal layer (data not shown). Application of highly purified OVA or peanut protein extract to the skin after removal of the stratum corneum induced high levels of antigen-specific IgG1 and IgE antibody but little or no IgG2a, which is characteristic of a Th2 response (Fig. 1A). No significant antibody response was detected following application of these antigens to skin if the stratum corneum had not been disrupted. Potent cell-mediated immunity following epicutaneous immunization was indicated by delayed-type hypersensitivity (DTH) responses elicited by injection of antigen into the footpad (Fig. 1B) and by *in vitro* proliferation of T cells from draining cervical LN (CLN) and spleens to the specific soluble antigens (Fig. 2A). Dose-dependent T cell proliferation to either OVA or peanut protein *in vitro* was detected 6, 10 and 20 days after the initial epicutaneous immunization. No proliferation to peanut or OVA was observed with T cells from mesenteric LN (MLN) of sensitized animals, which is consistent with the T cell activation occurring by immunization through the skin and not inadvertently by ingestion. When antigen was applied to intact skin that had not had the stratum corneum removed, detectable but much lower levels of T cell proliferation were observed.

Cytokine production by T cells from draining CLN stimulated with antigen *in vitro* was also determined (Fig. 2B). IFN- $\gamma$  production by T cells taken 6 days after the initial epicutaneous immunization was relatively high reaching 3,500 pg/ml, but the level decreased significantly after 10 days and was <600 pg/ml after 20 days. In contrast, IL-4 levels were low (25 pg/ml) but significantly above background 6 days after the initial epicutaneous immunization and increased substantially reaching on average of 160 pg/ml after 20 days. Essentially the same pattern of cytokine production was observed with splenic T cells, although the levels were lower. MLN T cells produced no detectable cytokines in response to either OVA or peanut protein, which is consistent with the absence of antigen-induced T cell proliferation.

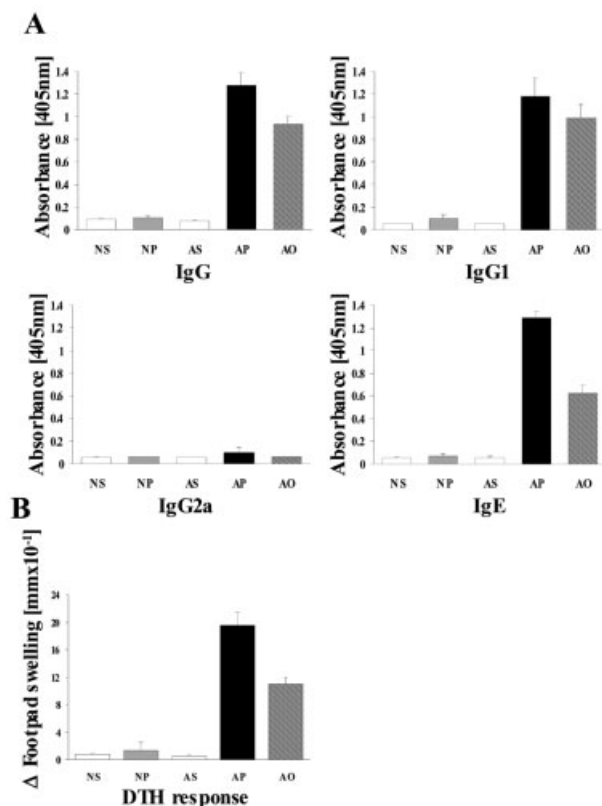


**Fig. 1.** Specific antibody (A) and DTH (B) responses in mice after epicutaneous immunization with peanut protein or OVA. Specific IgG, IgG1, IgG2a and IgE in serum diluted ten-fold in PBS 20 days after the initial epicutaneous immunization were measured by ELISA to the immunizing antigen and expressed as mean absorbance  $\pm$  1 SEM ( $n \geq 6$ ). DTH responses were measured 24 h after single peanut protein or OVA challenge without adjuvant in the footpad 20 days after the initial epicutaneous immunization, and expressed as mean increment of footpad swelling  $\pm$  1 SEM ( $n \geq 6$  group). Similar results were obtained from eight individual experiments. NS: Normal skin painted with saline; NP: normal skin painted with peanut; AS: abraded skin painted with saline; AP: abraded skin painted with peanut; AO: abraded skin painted with OVA.

## 2.2 Epicutaneous compared with subcutaneous immunization

The importance of the route of immunization was investigated by comparing epicutaneous with subcutaneous immunization. In these experiments, peanut protein was applied three times to the skin after removal of the stratum corneum or injected subcutaneously into the dermis without adjuvant at the base of each ear for 3 consecutive days. As expected, T cells from draining CLN and spleens of mice immunized by the epicutaneous route showed significant antigen-specific proliferation, but there was very little proliferation by T cells from mice

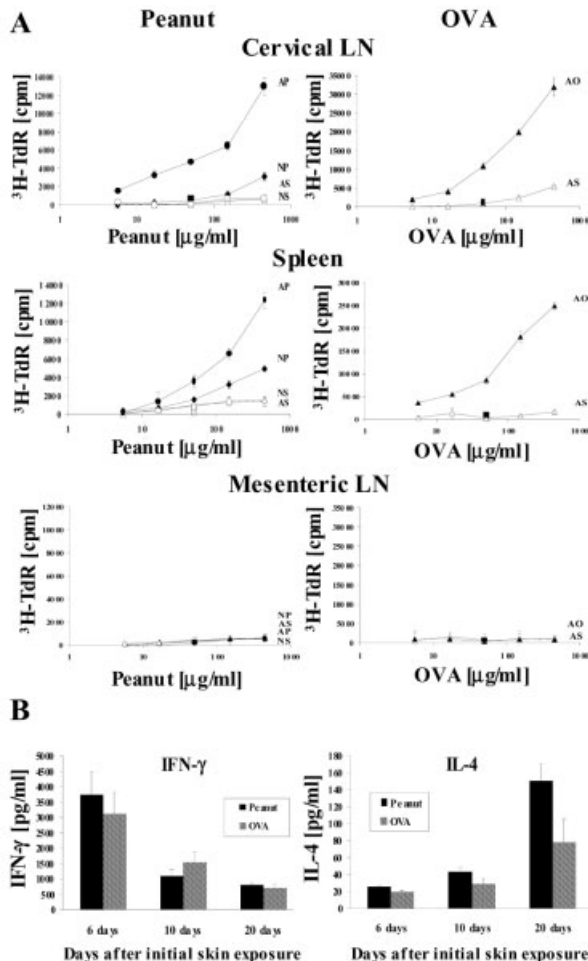


**Fig. 2.** Antigen-specific T cell proliferation and cytokine production. Pooled CLN, MLN and splenic lymphocytes from 6 mice were cultured with antigen 6 days after the initial epicutaneous immunization (A) and proliferation measured by <sup>3</sup>H-thymidine incorporation. Results are expressed as mean cpm from triplicate cultures  $\pm$  1 SEM. Background proliferation from cells cultured without antigen has been subtracted. Responses shown are by T cells from mice immunized with peanut protein (AP) or OVA (AO) on abraded (● and ▲), or with peanut protein (NP) on normal (◆) skin and mice painted with saline on abraded AS (△) or normal NS (○) skin. Challenge with an irrelevant antigen is shown (■). The experiment was repeated eight times with similar results. IFN-γ and IL-4 production by T cells from draining CLN taken 6, 10 and 20 days after the initial epicutaneous immunization was determined after 96 h culture with appropriate antigen (B). Results are expressed in mean pg/ml  $\pm$  1 SEM of between two and eight individual experiments. No cytokines were produced in response to an irrelevant antigen or when cells were cultured without antigen, and no cytokine production was detected by CLN T cells from control animals with abraded skin painted with saline.

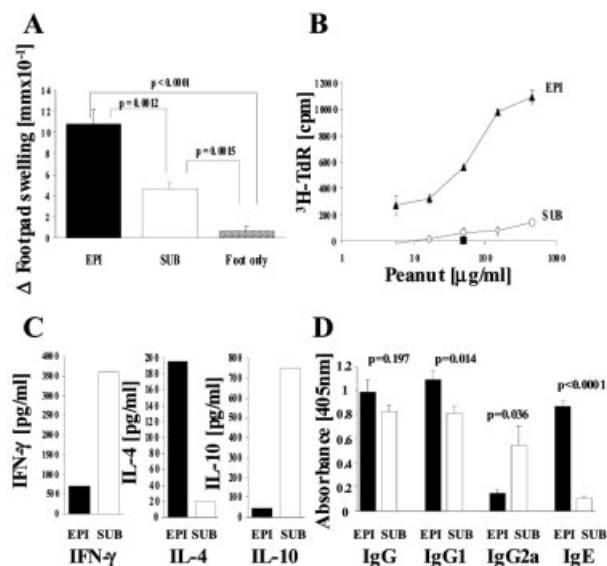


**Fig. 3.** Comparison between epicutaneous and subcutaneous immunization. Mice were immunized epicutaneously (EPI) or subcutaneously (SUB) with peanut protein. DTH responses were determined 24 h after single peanut challenge in the footpad 20 days after the initial epicutaneous or subcutaneous immunization (A). T cells taken from draining CLN 20 days after the initial epicutaneous ( $\blacktriangle$ ) or subcutaneous ( $\circ$ ) immunization were cultured for 96 h with peanut protein or an irrelevant antigen ( $\blacksquare$ ) and proliferation determined by [ $^3\text{H}$ ]thymidine incorporation (B). IFN- $\gamma$ , IL-4 and IL-10 production by CLN T cells cultured for 96 h with peanut protein was measured by ELISA (C). Peanut-specific IgG, IgG1, IgG2a and IgE was measured in serum 20 days after the initial epicutaneous or subcutaneous immunization. Sera were diluted tenfold prior to analysis and the results are expressed as mean absorbance  $\pm$  1 SEM from six mice (D).

immunized by the subcutaneous route (Fig. 3). A marked DTH response was obtained in mice immunized by the epicutaneous route and a lesser but nevertheless significant DTH response was also obtained after sensitization by subcutaneous immunization. These results show that the epicutaneous route generates a more potent T cell immune response than subcutaneous immunization.

T cells from draining CLN 20 days after epicutaneous immunization produced only small amounts of IFN- $\gamma$  or IL-10 and large amounts of IL-4. In contrast, T cells from mice immunized subcutaneously produced high levels of IFN- $\gamma$  and IL-10 but only low levels of IL-4. This Th2 bias in mice immunized by the epicutaneous route and Th1 bias in mice immunized subcutaneously was also evident in the antibody response (Fig. 3D). The epicutaneous route induced high levels of IgG1 and IgE with little or no detectable IgG2a, whereas subcutaneous immunization resulted in much less antigen-specific IgG1 and IgE but high levels of IgG2a. These results are consistent

with epicutaneous immunization generating a predominantly Th2 response, whereas subcutaneous immunization elicits a predominantly Th1 response.

### 2.3 Responses by different mouse strains

To exclude the possibility that the effectiveness and Th2 bias of epicutaneous immunization were peculiar to BALB/c mice, responses in two other mouse strains (C57BL/6 and CBA/C9) were investigated. These two strains responded in much the same way as BALB/c mice. High levels of specific IgG1 and IgE but little IgG2a were produced, and T cells from draining CLN but not MLN proliferated well to the relevant recall antigen and produced high levels of IL-4 but low levels of IFN- $\gamma$  (Fig. 4). Significant DTH responses were also elicited in both mouse strains (results not shown).

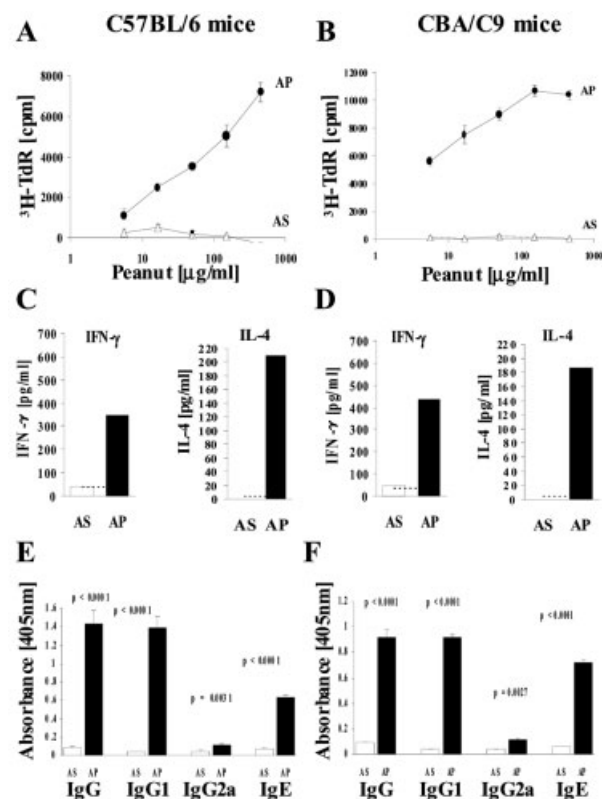


**Fig. 4.** Response of different mouse strains to epicutaneous immunization. C57BL/6 (A, C, E) and CBA/C9 (B, D, F) mice were immunized epicutaneously with peanut protein ( $n=6$ ). T cell proliferation (A, B) and cytokine production (C, D) was determined as for BALB/c mice described in Fig. 2. Specific IgG, IgG1, IgG2a and IgE production was determined as for BALB/c mice described in Fig. 1. AS: Skin abraded and painted with saline; AP: skin abraded and painted with peanut protein.

## 2.4 Removal of the stratum corneum alters LC morphology and maturation status

Removal of the stratum corneum in the procedure used here for epicutaneous immunization directly exposes the LC in the surface epidermis to antigen without disruption of the normal skin architecture. The effects of the tape-stripping technique on LC were therefore investigated. Epidermal sheets were prepared from the ear and LC morphology and maturation markers examined. Epidermal LC were visualized in the normal epidermis as large MHC class II<sup>+</sup> cells with long dendrites extending out from a spindly cell body, creating a brightly stained contiguous network on top of the dimly MHC class II<sup>+</sup> keratinocytes (Fig. 5). Within 2 h of removing the stratum corneum, LC morphology began to change as dendrites were gradually pulled in and the cell body widened. By 12 h, the cells were rounded with few visible dendrites and by 24 h the morphology of the LC had changed completely to rounded or oval cells with a ruffled appearance. They also stained more brightly for MHC class II (Fig. 5). By 48 h, LC morphology and the appearance

of the epidermis as a whole returned to normal (not shown).

The changes in LC morphology and the increased expression of surface MHC class II is consistent with the well-documented maturation process of DC [3]. To investigate this process further, the expression of costimulatory and maturation markers on LC in normal intact skin and in skin 24 h after removal of the stratum corneum was examined. Freshly prepared epidermal sheets were double stained for MHC class II and either CD86, CD40, CD54 or CD11c and examined by confocal microscopy. In normal skin with an intact stratum corneum, the expression of CD86, CD40, CD54 and CD11c was very low or not detectable (Fig. 5B). In contrast, within 24 h after removal of the stratum corneum, the expression of all of these molecules on LC was significantly enhanced. The *in situ* epidermal keratinocytes did not express CD86, CD40, CD54 or CD11c in either normal or abraded skin.

## 2.5 LC migration to draining LN

Despite the changes in LC morphology and surface expression, removal of the stratum corneum did not cause them to migrate. In contrast, analysis of epidermal sheets after removal of the stratum corneum and exposure to antigen revealed a rapid migration of LC from the epidermis (Fig. 6). Within 2 h of epicutaneous antigen

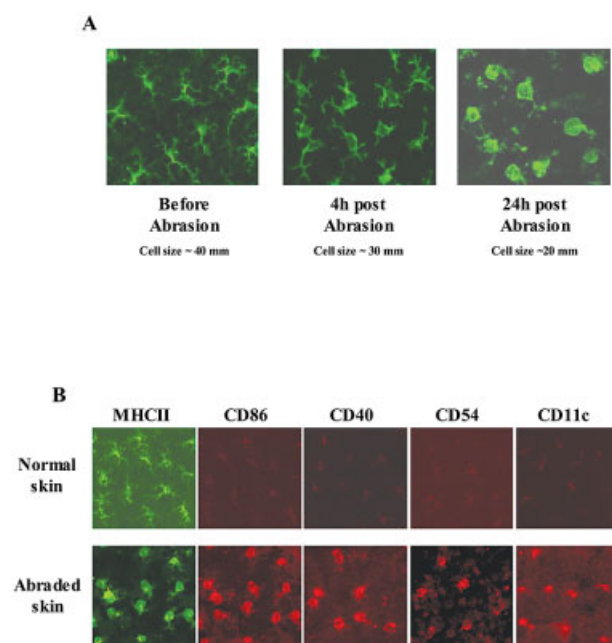


**Fig. 5.** Effect of removing the stratum corneum on LC morphology and surface antigen expression. Confocal images of skin epidermal layers stained for MHC class II (FITC) before, and 4 and 24 h after removal of the stratum corneum (A), and for MHC class II (FITC), CD86<sup>+</sup>, CD40<sup>+</sup>, CD54<sup>+</sup> and CD11c<sup>+</sup> (TRITC) before and 24 h after removal of the stratum corneum (B). Labeled cells are LC. Epithelial cells (keratinocytes) can be seen in the background as a 'cobblestone' pattern of faintly MHC class II<sup>+</sup> cells. Original magnification  $\times 100$ .

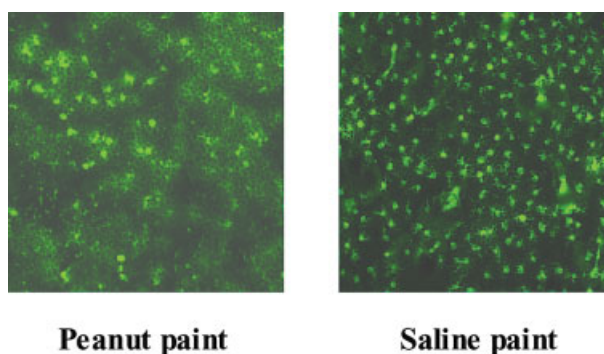


**Fig. 6.** Effect of antigen on LC migration from the epidermis. Confocal images of epidermal sheets 2 h after epicutaneous exposure to antigen or saline on abraded skin. Reduced numbers of MHC class II-staining LC in the epidermis after exposure to peanut protein compared to saline can be seen, indicating migration from the skin in response to antigen. Mean LC counts in defined visual grids of the epidermis  $\pm 1$  SEM when the skin was exposed to peanut were  $40 \pm 5$  compared to  $268 \pm 29$  per  $\text{mm}^2$  when exposed to saline alone,  $p < 0.05$ . LC density in normal naive skin was  $293 \pm 32$ . Note the 'cobblestone' pattern of the MHC class II<sup>+</sup> keratinocytes. Original magnification  $\times 20$ .

exposure the majority of LC had migrated away from the epithelial layer, while only minor migration was observed in response to saline alone ( $p < 0.05$ ). Some repopulation of LC occurred within 14 h, but there were still significantly fewer visible in the epidermis at this time compared to controls. Complete repopulation of the epidermis with a normal network of immature LC displaying extended dendrites did not occur until 48 h after antigen exposure (data not shown).

To follow LC migration after exposure to antigen, FITC-conjugated peanut protein was applied to normal skin or to skin after removal of the stratum corneum. LC that migrated to the draining LN were identified by flow cytometry as CD11c<sup>+</sup> cells that had taken up FITC-conjugated peanut protein. The number of CD11c<sup>+</sup> DC in the draining LN increased from a background level of approximately 12% of gated cells to nearly 20% after application of antigen on abraded skin ( $p < 0.05$ ) (Fig. 7), suggesting significant migration of CD11c<sup>+</sup> cells from the epidermis only following abrasion and antigen exposure. This was consistent with the histology. A large increase in double-stained CD11c<sup>+</sup>/FITC<sup>+</sup> cells was also observed, showing that LC had taken up FITC-conjugated peanut protein and migrated from the epithelial surface to the draining LN. This did not occur when FITC-conjugated peanut protein was applied to intact skin or when abraded skin was exposed to saline alone ( $p < 0.0005$ ).

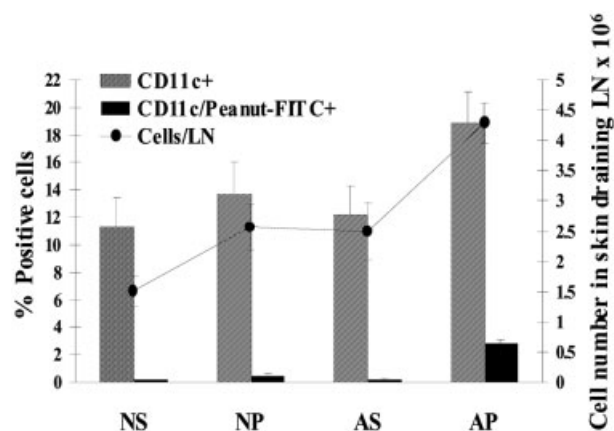


**Fig. 7.** LC migration from the epidermis to draining LN following epicutaneous exposure to antigen. FACS analysis of LC (CD11c<sup>+</sup>) and LC that have taken up peanut protein (CD11c<sup>+</sup>/peanut-FITC<sup>+</sup>) in draining CLN cells 18 h after a single epicutaneous application of FITC-conjugated peanut or saline to intact skin (NP and NS, respectively) or to skin after removal of the stratum corneum (AP and AS, respectively). Total number of cells in CLN is also shown (●). Mean counts  $\pm$  1 SEM of four separate experiments are shown.

### 3 Discussion

The incidence and severity of allergic disorders has steadily increased in the industrialized world over the past three decades. Most of these diseases are associated with an increased and sustained allergen-specific IgE production, which mediates immediate type I allergies upon re-exposure to the specific allergen. The mechanism of primary sensitization to protein allergens is clearly important in pathogenesis of the disease. In young children, the skin is often exposed to food antigens. In addition, many cosmetics, lotions and bath additives contain vegetable oils including peanut oil with small amounts of protein allergens. Children with atopic dermatitis frequently have various ointments, often containing vegetable oils, applied to the affected skin areas to control disease. The use of peanut-containing creams has been shown to be more widespread in children who have become allergic to peanut than in control infants [19].

The current study was initiated to investigate the immune responses obtained by skin exposure to common high molecular-weight protein antigens. The stratum corneum was disrupted in these experiments to mimic the desquamated skin of atopic dermatitis and other inflammatory skin conditions. Application of OVA or partially purified peanut protein to skin after removal of the stratum corneum elicited a potent systemic immune response. Both the cell mediated- and antibody responses obtained were predominantly Th2, as indicated by increased IL-4 and reduced IFN- $\gamma$  production by T cells from draining LN, and by high levels of IgG1 and IgE antibody and little or no IgG2a. In contrast, the response elicited by subcutaneous immunization was predominantly Th1. Other studies have reported that prolonged application of OVA to shaved murine skin can result in elevated levels of serum IgE [16, 17]. In these studies, however, OVA was applied to shaved skin under an occlusive patch over a period of total 7 weeks. This method may have caused some abrasion by shaving, induced immunological changes in the skin and aid penetration of proteins as a result of local dilation of blood vessels. Other skin immunization protocols have used lipid solvents or adjuvants such as cholera toxin [20]. In contrast, our results clearly show that a systemic Th2 immune response can be obtained by single applications of antigen to skin that has had the stratum corneum removed by simple adhesive tape stripping and without occlusion or addition of solvents or adjuvants. The high level of IgE produced by this route of immunization may result in primary allergic sensitization. Impaired barrier-function of the epidermis even in areas of skin without lesions is characteristic of atopic dermatitis [18, 21]. It is also possible that excessive washing with rough materi-

als or using syndets and other de-fattening agents and/or frequent use of adhesive tape found for example on disposable nappies may cause abrasion of the skin of babies and young children and promote allergic sensitization to food and other environmental antigens.

The method of skin abrasion used here was shown to remove the stratum corneum exposing the epidermis and LC without disrupting the living epidermal layer. Application of antigen to the abraded skin gave direct access to the epidermis and induced potent and Th2-biased immunity. In contrast, subcutaneous immunization delivered antigen directly into the dermis and gave rise to Th1-type immune responses. This suggests that primary antigen exposure to LC favors the induction of Th2 immunity. It has been shown that T cells grown with cultured LC make IL-4 and stimulate IgE production by B cells consistent with a Th2 bias by this route of antigen presentation [22]. Other immunological sites at body surfaces have also been reported to induce predominantly Th2 responses when challenged with protein antigen without adjuvant. In the mouse, exposure of the lung to aerosolized OVA without adjuvant results in IgE production and airway hyper-responsiveness [23]. Furthermore, resting DC in the respiratory tract preferentially stimulate Th2 responses and require additional cytokine signals for induction of Th1 immunity [24]. Intranasal exposure to OVA has also been shown to induce Th2-type responses with IgE production [25, 26]. DC residing at the body surfaces may also have a specialized role by inducing tolerance rather than immunity. Gut lamina propria DC usually reside in an immature state, lacking a full range of costimulatory molecules such as CD80/CD86, CD40 and CD54. These cells may be tolerogenic if they present antigen in absence of inflammation [27, 28]. Proliferation but not maturation of DC *in vivo* with Flt3 ligand (Flt3L) enhances the induction of oral tolerance [28], while simultaneous proliferation and maturation of DC *in vivo* with Flt3L and IL-1 $\alpha$ , indicated by expression of high levels of MHC class II and costimulatory molecules, abrogates the induction of oral tolerance and active immunity is achieved [29]. It is thus tempting to speculate that LC and other DC found on body surfaces have an inherent or default mechanism to promote tolerance and/or anti-inflammatory Th2 responses to dampen down Th1 responses that may be elicited by harmless symbiotic microbes found on skin and in the gut. Inappropriate Th1 immune responses elicited at body surfaces may cause injury, allowing microbes to gain access to deeper layers.

In addition to the natural phenotype of LC in the epidermis, the local response to cutaneous barrier disruption may play a role in the Th2 dominance of the immune response after epicutaneous antigen exposure. The response to barrier disruption may result in cytokine pro-

duction or expression of other molecular signals in the skin that promote Th2 responses. IL-10 is absent in normal intact skin, but it is expressed in the epidermis soon after barrier disruption [7, 30], and may play a role in suppressing Th1 cytokine production. IL-4 is expressed in skin after barrier disruption and exposure to antigen [18, 31], and may alter LC function to promote Th2 responses. IL-4 has been shown to make LC more efficient in taking up and processing naive protein [32]. GM-CSF is also produced in response to barrier disruption [5] and either alone or in combination with IL-4 augments the rate of pinocytosis by precursor DC [33]. Additionally, IL-13, which is highly expressed in atopic dermatitis skin lesions, has been shown to be critically important for Th2 generation in the skin and can substitute the need for IL-4 [34, 35]. In our experiments, removal of the stratum corneum increased MHC class II, CD86, CD40, CD54 and CD11c expression on LC (Fig. 5). Barrier disruption itself, however, was not sufficient to cause LC to migrate to the draining LN (Fig. 6, 7), but significant migration was observed when antigen was applied to the barrier-disrupted skin. The migration was not likely induced by LPS contamination of the peanut protein preparation, as no LPS could be detected by limulus assay. This strongly suggests that maturation of LC defined by increased expression of MHC class II, CD86, CD40, CD54 and CD11c is not sufficient for migration and additional signals are required. A similar conclusion was reached in two recent *in vitro* studies showing that maturation of human monocyte-derived DC did not induce migration and that a second PGE<sub>2</sub> signal was required [36, 37]. Other studies have also indicated that LC maturation and migration can be independently regulated events [38].

This report has shown that protein antigen can easily enter through abraded skin and cause primary Th2-biased immune responses. The results suggest that the activated LC in the epidermis play a pivotal role in promoting the Th2 responses. The evidence from this and other studies thus demonstrate that the skin is an active immunological site and that epicutaneous allergen exposure should be considered when therapeutic regimens for atopic diseases are developed. Restoration of cutaneous barrier function may be a potential target for immunotherapeutic intervention to correct the cytokine imbalance in atopic dermatitis, and may thus circumvent some allergic and DTH responses.

The effectiveness of epicutaneous immunization makes the skin a potentially attractive non-invasive route for vaccine delivery. There has recently been some interest in exploiting the immune system of the skin for vaccine administration, and strategies for 'transcutaneous immunization' have been developed. These new strategies rely on strong nonspecific adjuvants, such as cholera

toxin and heat-labile enterotoxin of *Escherichia coli*, co-applied with the antigen on the skin for good immunity to high-molecular weight proteins [20, 39, 40]. However, the inclusion of such toxins is not generally acceptable for human vaccines. In contrast, the simple abrasion method described here generates effective immune responses without the use of adjuvants or toxins. Inclusion of agents that promote Th1 responses may allow for modulation of the dominant Th2 response, but this has yet to be tested. The possibility of using skin abrasion and *in situ* LC activation as a natural vaccine adjuvant in humans is currently being explored in our laboratories in a pilot clinical trial. The Th2 dominance of responses to epicutaneous immunization may additionally have direct therapeutic applications in Th1 diseases, such as collagen-induced arthritis, diabetes and EAE, which is being investigated using murine models of Th1-type autoimmune disease.

## 4 Materials and methods

### 4.1 Mice

Female BALB/c, C57BL/6 and CBA/C9 mice aged 6–8 weeks were used in this study. They were kept under specific pathogen-free conditions and fed on a special diet free of peanut, OVA, soy and cows milk. Water was available *ad libitum*.

### 4.2 Protein antigens

Partly defatted peanut flour was obtained from the Golden Peanut Company (Alpharetta, GA). A concentrated peanut protein extract was prepared using a modification of the method described by de Jong et al. [41]. In brief, the flour was defatted with hexane five times and then extracted with 0.1 M  $\text{NH}_4\text{HCO}_3$ . The supernatant was removed and precipitated with 60%  $(\text{NH}_4)_2\text{SO}_4$  for 2 h at 4°C. The precipitate was centrifuged, dissolved in a minimum volume of PBS and dialyzed extensively against PBS. This method uniformly yielded 85–90% pure peanut protein. LPS content was determined using E-toxate® endotoxin reagent limulus polyphemus (Sigma, GB) according to manufacturer's instructions. The peanut powder routinely contained <0.05–0.1 EU/ml LPS, which was the detection limit of the assay. OVA grade V was purchased from Sigma. Peanut protein and OVA were biotinylated and FITC-conjugated using standard methods [42].

### 4.3 Epicutaneous and other immunizations

The stratum corneum was removed from both sides of the earlobe by application and removal of cellophane tape (Scotch™) five to eight times. Twenty-four hours later, 25 µl

of OVA or peanut protein in PBS (4 mg/ml) were applied to both sides of the earlobe with a cotton bud. A maximum of 100 µg protein was deposited on each side of the ear by this technique. The application of protein to the skin was repeated on the next 2 consecutive days. Antigen applied to intact skin that had not been tape-stripped, and PBS without antigen applied to stripped skin and intact skin were included as controls. Other mice were immunized with 100 µg protein in PBS injected subcutaneously into the dermis at the base of each ear on 3 consecutive days. At 6, 10 or 20 days after the initial skin immunization the mice were killed and the draining CLN, MLN, spleen and serum collected.

### 4.4 DTH response

To elicit a DTH response, mice were challenged 20 days after the last epicutaneous or subcutaneous immunization by injection of 100 µg protein in PBS into the left hind footpad. Net footpad swelling was measured using a microcalliper (Mitutoyo, Japan) 24 h after challenge. Mice were killed immediately after the 24 h footpad measurement.

### 4.5 T cell proliferation and cytokine production

Spleen and LN cell suspensions were obtained by mechanical disaggregation, and  $2 \times 10^5$  cells cultured in 96-well flat-bottom plates in a total volume of 200 µl RPMI 1640 medium supplemented with 10% FCS, 50 µM 2-ME and 5 µg/ml gentamycin. OVA or peanut protein were added at concentrations ranging from 5 to 450 µg/ml. Control responses to the irrelevant antigen or concanavalin A at 1 µg/ml were also determined. Cultures were incubated at 37°C for 90 h and pulsed with 1 µCi [ $^3\text{H}$ ]thymidine (Amersham Pharmacia, GB) for the last 16 h. Cells were harvested and thymidine incorporation determined by liquid scintillation counting on a MicroBeta (Wallac, GB). Supernatants were assayed by ELISA for IL-4, IL-10 and IFN-γ using antibodies from PharMingen (San Diego, CA), according to the manufacturer's protocol. Recombinant mouse IL-4, IL-10 and IFN-γ from PharMingen were used as standards. The detection limit of the assays was 5 pg/ml for IL-4 and 40 pg/ml for IL-10 and IFN-γ.

### 4.6 Antibody responses

At the end of each experiment, mice were bled by cardiac puncture and sera prepared for specific antibody determinations. For IgG, IgG1 and IgG2a antibodies, 96-well Maxosorb plates (Nunc, Denmark) were coated with peanut or OVA protein at 250 µg/ml in carbonate-bicarbonate buffer at 4°C overnight. Appropriately diluted sera (100 µl in PBS) were added and the plates incubated at 37°C for 90 min. After washing, alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG Fc (Sigma), rat mAb to mouse IgG1

(Zymed, San Francisco, CA) or rat mAb to IgG2a (PharMingen) were added for 1 h at 37°C. The alkaline phosphatase substrate pNPP (Sigma) was then added and absorbance measured at 405 nm. Antigen-specific IgE was measured by an IgE capture method. Sera to be tested were added to Maxisorb microtiter plate wells coated with 1 µg/ml rat monoclonal anti-mouse IgE (PharMingen). Biotinylated peanut or OVA were then added at a concentration of 100 µg/ml and incubated for 2 h at 37°C. After washing, alkaline phosphatase-streptavidin (PharMingen) was added for 1 h followed by pNPP substrate.

#### 4.7 Immunohistology

Epidermal sheets were prepared from mouse ears that had been split into dorsal and ventral sides using forceps to pull the two layers apart. The skin was incubated in 20 mM EDTA for 4 h at 37°C. Epidermal sheets were gently lifted from the dermis and subcutaneous fat, washed in PBS and fixed in cold acetone for 20 min. at –20°C. After washing in PBS, the epidermal sheets were first incubated in 2% BSA/PBS for 1 h at room temperature and then stained with monoclonal rat anti-mouse I-A<sup>d</sup>/I-E<sup>d</sup>, CD86, CD40, hamster anti-mouse CD54 (PharMingen), hamster anti-mouse CD11c (Endogen, Cambridge, MA) or isotype control antibody at 4°C overnight. They were then washed thoroughly in PBS and bound antibody detected with FITC-conjugated rat anti-mouse Ig (DAKO, Denmark), TRITC-conjugated donkey anti-rat Ig (Jackson, West Grove, PA) or TRITC-conjugated goat anti-hamster Ig (Jackson) in 1% BSA/PBS for 90 min. at 37°C. Epidermal sheets stained for CD86, CD40, CD54 and CD11c were counterstained with FITC-conjugated antibody to I-A<sup>d</sup>/I-E<sup>d</sup> for 90 min at 37°C. After extensive washing, epidermal sheets were mounted onto slides with aquamount and examined using a Leica SP2 confocal laser scanning microscope system (Leica NT, Milton Keynes, GB). The numbers of stained cells in at least 20 visual fields per epidermal sheet were counted using a grid of known area.

#### 4.8 LC migration

LC migrating from epidermis to draining LN were identified by uptake of FITC-labeled antigen. At 24 h after removal of the stratum corneum by tape stripping, 100 µg FITC-conjugated peanut in PBS was applied to both sides of the ear. Cell suspensions of draining CLN were prepared 18 h after application of antigen using 0.4 mg/ml Liberase (Boehringer Mannheim, GB) for 25 min at 37°C to gently digest LN before filtering through a nylon mesh. Single-cell suspensions were stained for CD11c (Endogen) to identify DC and LC and analyzed on a FACSCalibur using CellQuest software (Becton Dickinson, GB).

#### 4.9 Statistical evaluation

The statistical significance of differences between experimental groups was determined using two-tailed Student's *t*-

test for unpaired data. Differences were regarded as significant when *p* < 0.05.

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