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Sensitization via healthy skin programmes Th2 responses in individuals with atopic dermatitis

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Abbreviations

FLG: filaggrin gene

AD: atopic dermatitis

ADWT: Atopic dermatitis without filaggrin mutations

ADFM: Atopic dermatitis with filaggrin mutations

DNCB: 2,4-dinitrochlorobenzene

Key words

Atopic dermatitis, filaggrin, Th2, skin, dendritic cell, DNCB

Abstract

Allergen-specific responses in AD are skewed towards a Th2 profile. However, individuals with AD have been shown to make effective virus-specific Th1 responses raising the possibility that the skin itself contributes to driving the AD Th2 immuno-phenotype. Therefore, to explore the programming of immunological sensitization by the skin, we examined the outcome of sensitization through non-lesional skin of AD and healthy controls.

Volunteers, (controls, AD with Filaggrin gene (*FLG*) mutations (ADFM) and AD without *FLG* mutations (ADWT)) were sensitized by cutaneous application of 2,4-dinitrochlorobenzene (DNCB), a small, highly lipophilic chemical sensitizer. At the doses tested, DNCB showed equal penetration into skin of all groups. Clinical reactions to DNCB were significantly reduced in AD. Although both controls and AD made systemic DNCB-specific Th1 responses, these were reduced in AD and associated with significantly Th2-skewed DNCB-specific T cell responses. Th2 skewing was seen in both ADFM and ADWT with no difference between these groups. After 3 months DNCB-specific Th2 responses were persistent in AD, and Th1 responses persisted in controls.

These data provide evidence that when antigen penetration is not limiting, AD skin has a specific propensity to Th2 programming suggesting the existence of altered skin immune signaling which is AD-specific and independent of *FLG* status.

Introduction

The immune responses of people with atopic dermatitis (AD) and atopic asthma are characterized by the generation of allergen-specific Th2 cells. Null mutations in the gene encoding filaggrin (*FLG*) occur in up to 50% of people with moderate to severe atopic dermatitis (AD) (Barnes, 2010; Palmer *et al.*, 2006). This finding has redirected interest in understanding the role of the epidermis in the initiation of AD which mirrors recent work suggesting the key role of defective bronchial epithelia in programming immune responses in atopic asthma (Xiao *et al.*, 2011). Data demonstrating that skin barrier function is defective in AD skin (Jakasa *et al.*, 2006; O'Regan *et al.*, 2010; Proksch *et al.*, 2006) and that impaired barrier function parallels disease severity (Nemoto-Hasebe *et al.*, 2009) confirm that skin barrier function is important, but this does not fully explain the immunophenotype of AD. The Th1/Th2 paradigm (Mosmann *et al.*, 1986; Romagnani, 2000; Umetsu *et al.*, 1988) has been extensively explored in atopic dermatitis (AD) and whereas we, and others, have shown that while allergen-specific Th1 cells can be detected in atopic and non-atopic individuals, allergen-specific Th2 cells are present in AD but not in non-atopic controls (Ardern-Jones *et al.*, 2007). A mouse model has recently provided evidence that homozygous mutations in *FLG* are implicated in Th2 skewing (Fallon *et al.*, 2009) and although genetic association between AD and the genes IL-4, IL-4R and IL-13 has also been widely replicated, the precise reason for the bias towards a cutaneous Th2 response in AD remains obscure. Indeed, individuals with AD are not systemically immunocompromised, make appropriate vaccine-specific Th1 memory cells (Schneider *et al.*, 2010), and have normal circulating Treg/Th17 frequency (Koga *et al.*, 2008; Maggi *et al.*, 2007). However, the fact that relative deficiency of Treg/Th17 cells has been identified in AD skin suggests that the primary immune defect in atopic dermatitis may be intrinsic to the epidermis (Guttman-Yassky *et al.*, 2008; Verhagen *et al.*, 2006).

Several lines of evidence point to the skin as being centrally involved in programming the atopic immune responses but up to now the definitive demonstration of this has been lacking. Thus, there is compelling evidence that the epidermal cytokine thymic stromal lymphopoietin (TSLP) can program dendritic cells via increased expression of OX40 to induce the generation of inflammatory Th2 cells characterized by the production of TNF- α in addition to the classical Th2 cytokines (Liu, 2006; Oyoshi *et al.*, 2010). It has also

been proposed that the Th2 allergen-specific responses observed in AD are a result of the defective barrier in the skin (O'Regan *et al.*, 2008) or the chemical properties of the proteins themselves (Shakib *et al.*, 2008). To address the question of whether primary epithelial regulation is critical in determining the quality of the allergic immune response, we used an experimental immunization system in humans delivered via the epidermis, and compared responses in AD and control subjects. Immunization or sensitization to environmental proteins would be unethical and results may be biased by epidermal barrier function. Previous studies of the histological features of cutaneous responses to the model sensitizer 2,4-dinitrochlorobenzene (DNCB) in pre-sensitized individuals, document spongiosis and perivascular mononuclear infiltrate arising at 48 hours which are the hallmarks of an eczematous response (Hartman *et al.*, 1976). Therefore, using DNCB which is not found in the environment, we could track the first exposure of an antigen through the skin and monitor the subsequent clinical and cellular immune priming of antigen-naïve individuals to an allergen whose penetration was unaffected by the structural integrity of the skin barrier.

Results

Penetration of DNCB is independent of FLG status

2,4-dinitrochlorobenzene (DNCB) has found wide use in studying contact sensitization as it is not present in the environment and can sensitize all individuals by cutaneous application (Friedmann *et al.*, 1983). Covalent DNCB haptenation of host proteins is a prerequisite for the induction of contact hypersensitivity; the DNCB modified proteins being processed and presented in both the class I and II MHC pathways to CD8 and CD4 T lymphocytes (Pickard *et al.*, 2007). Consequently, tracking T cell sensitization to DNCB gives a unique view of the initiation of immune responses generated by percutaneous antigen exposure in humans. DNCB is a low molecular weight (202 Da) highly lipophilic molecule that penetrates the skin efficiently independent of skin structural barrier function (Falconer *et al.*, 1992; Gawkrödger *et al.*, 1989; Tsai *et al.*, 2001). Equivalent penetration of DNCB at sensitizing the dose in AD and controls is critical to this study therefore we firstly set out to confirm this assumption. Using the established methodology developed by Franz (Franz *et al.*, 2009) we tested penetration of DNCB in human explant skin. In subsequent clinical studies we used a sensitizing dose which has been shown to sensitize 100% of individuals ($60\mu\text{g}/\text{cm}^2$). However, to avoid missing subtle alterations in penetration, we used only $20\mu\text{g}/\text{cm}^2$ in penetration assays, which would only sensitize approximately half the population (Friedmann *et al.*, 1983). Following overnight penetration of DNCB through the surface of skin biopsies from healthy controls, individuals with atopic dermatitis without *FLG* mutations (ADWT) or individuals with atopic dermatitis with *FLG* mutations (ADFM), anti-DNCB fluorescence staining of histological sections was used to assess penetration of the antigen (Fig. 1a-f). Although we cannot extrapolate these data to lower doses of DNCB, as expected, at one third of the dose used in subsequent clinical experiments, equivalent strong penetration to the basement membrane was identified histologically between controls, ADWT or ADFM and the relative fluorescence of anti-DNCB at the outermost viable epidermal level (base of stratum corneum) compared to the basal keratinocyte layer showed no significant difference between controls, ADWT or ADFM (Fig. 1h).

Circulating lymphocytes in AD show a normal Th1/2 ratio

A Th2 polarized repertoire of circulating T cells in AD would be likely to modify immune priming towards Th2 and thus would complicate the investigation of the epithelial regulation of immune responses. Therefore we first characterized the Th1/2 balance of circulating T cells in AD and controls in our population. Firstly, we compared the production of IL-4 and IFN- γ by ex-vivo PBMC from AD (n=18) and healthy controls (n=10) and confirmed that resting unstimulated cells showed no difference in basal production of IL-4 (p=0.37) or IFN- γ (p=0.94) (data not shown). We then compared non-T cell receptor (TCR) mitogen-induced T cell activation (phytohemagglutinin, PHA) against a direct TCR signal delivered by Staphylococcal enterotoxin B (SEB). SEB cross-links specific SEB-binding TCR-V β chains to MHCII thereby inducing T cell activation in a subset of responding T cells. *In vitro* activation showed that there was no significant difference in Th1 responses between PBMC from both AD and controls when stimulated by PHA or SEB: p=0.06; p=0.1 respectively (Fig. 2). Importantly, circulating T cells in both AD and controls showed a similar frequency of Th2 polarized cells with no statistically significant difference in frequency or ratio between the groups (Fig. 2). PBMC responses to House Dust Mite showed the expected Th2 programming in AD which was absent in controls (Fig 2e). Therefore it seems likely that the Th2 polarized responses to environmental proteins characteristic of AD are contained within a very small fraction of circulating lymphocytes and because of such low frequency are unlikely in themselves to perpetuate Th2 programming.

AD associates with impaired allergic contact dermatitis responses to DNCB

One month after sensitization, participants were re-challenged on a different non-eczematous site by epicutaneous application of a dose series of DNCB as we have previously described (Pickard *et al.*, 2009). In concordance with previous findings (Rees *et al.*, 1990), individuals with AD showed significantly reduced contact hypersensitivity responses to DNCB compared to healthy controls. This was reflected both by reduced inflammatory edema (skinfold thickness) (Fig. 3 a, p=0.007) and erythema (Fig. 3b, p=0.015). Calculation of the area under the dose response curve (AUC) in each of healthy controls, ADFM and ADWT, demonstrated that compared to healthy controls, reduced cutaneous reactivity (skin fold thickness and erythema) was evident in both ADWT and ADFM (Fig. 3c, d). ADFM showed further reduced skin

fold responses to DNCB challenge as compared to ADWT but this difference did not reach statistical significance ($p = 0.44$, Fig. 3c).

DNCB-specific Th1 and Th2 responses

To demonstrate that circulating DNCB-specific T cells could be characterized in DNCB-sensitized individuals we firstly confirmed by ELISpot and flow cytometric double cytokine intracellular staining that both Th1 (Fig. 4a, c) and Th2 (Fig. 4d, f) DNCB-specific T cell lines could be expanded in culture from the PBMC of sensitized individuals to high frequencies. As expected, IFN- γ producing DNCB-specific T cells were not able to simultaneously produce IL-4 (Fig. 4c). Similarly, IL-4-specific T cells were unable to produce IFN- γ (Fig. 4f).

Sensitization through the skin induces skewing towards antigen-specific Th2 responses in AD

To examine the cytokine polarization of antigen-specific T cells following cutaneous sensitization with DNCB, PBMC from patients and controls were tested *ex-vivo* for DNCB-specific IFN- γ and IL-4 production by ELISpot assay (Fig. 5a,b). No IFN- γ or IL-4 DNCB-specific responses were detected in non-DNCB sensitized controls (Fig. 5a,b). Although both groups demonstrated Th1 DNCB-specific responses (Fig. 5b), induction of DNCB-specific Th2 responses were largely confined to AD individuals ($p=0.02$) (Fig. 5a). Antigen-specific Th2 responses were significantly more frequent in both ADWT and ADFM ($p=0.02$ and 0.001 , respectively, Fig. 5c). ADFM showed a greater frequency of Th2-polarised DNCB-specific T cells compared to ADWT, however, this did not reach statistical significance ($p=0.59$; Fig. 5c).

Long-lived DNCB-specific Th2 memory cells in AD.

Examination of the cohort 3 months after the re-challenge (4 months post sensitization) showed that DNCB-specific Th2 memory responses persist in individuals with AD (Fig. 5d). However, memory-Th1 responses are diminished in the AD group at this stage, which is in contrast to healthy controls who maintain Th1-memory responses to DNCB (Fig. 5e) although these differences were not statistically significant.

Discussion

The important role of filaggrin-mediated epithelial barrier dysfunction in mediating AD is assumed but the same genetic defect is also present in some healthy individuals without AD (9% European population (Palmer *et al.*, 2006)) and Ichthyosis Vulgaris patients without AD (Smith *et al.*, 2006). Indeed, it has been established that epidermal barrier function is defective in both eczematous and clinically normal skin of individuals with AD and application of allergens (e.g. house dust mite proteins) to the skin can induce skin lesions (Darsow and Ring, 2000; Ring *et al.*, 2001) which are indistinguishable clinically and microscopically from AD (Langeveld-Wildschut *et al.*, 1996; Langeveld-Wildschut *et al.*, 1995) thus supporting an important role for allergic sensitization in AD pathogenesis. Furthermore, allergen avoidance has been shown by some to be at least partially effective in the treatment of AD (Clark and Adinoff, 1989; Friedmann, 1999; Fukuda *et al.*, 1991; Sanda *et al.*, 1992; Tan *et al.*, 1996). Dysregulation of adaptive immunity in AD is reflected by Th2 biased allergen-specific responses which are critical to B cell class-switching and induction of allergen-specific IgE production. We have confirmed that circulating Der p 1-specific Th2 cells are only present in atopic individuals including AD whilst Der p 1 specific Th1 cells are detectable in both atopic subjects and healthy controls (Ardern-Jones *et al.*, 2007). This suggests that, although allergen has been encountered by both groups in a setting that initiates expansion of allergen-specific T cells, the immune response is differently skewed in individuals with atopy and AD. In the atopic march described in children, AD usually arises first, most frequently in the absence of IgE sensitization (Illi *et al.*, 2004; Kay *et al.*, 1994), and is a major risk factor for the development of severe asthma and food allergy (Illi *et al.*, 2006; Sporik *et al.*, 1990). The development of specific IgE to environmental allergens is associated with defective skin barrier function (Boralevi *et al.*, 2008) with skin-related sensitization contributing to the association between AD and childhood asthma as well as its persistence into adulthood (Burgess *et al.*, 2008). Clearly, understanding the molecular pathway which drives Th2 polarization which in turn induces allergen-specific IgE production in individuals with AD is critical to further development of therapeutic approaches for AD and potentially for atopic disease in general. Some previous studies have shown deficient Th1 pathways in circulating antigen presenting cells / monocytes (IL-12) in AD but others have shown opposing results (Itazawa *et al.*, 2003; Piancatelli *et al.*, 2008).

We therefore set out to explore the immunological outcome of the same antigenic stimulus in the skin of individuals with AD versus non-atopic controls. As a chemical sensitiser DNCB has a number of disadvantages as a model allergen for AD because it may not replicate the cutaneous response to protein allergens. However, DNCB reliably sensitizes all individuals and importantly, in contrast to proteins, skin penetration at the sensitizing dose used here, is not modified by AD or *FLG* status (Fig. 1) allowing us to test epidermal regulation of immune responses between AD and controls without being confounded by disease status. Furthermore, DNCB-haptenated proteins require intracellular processing for presentation at the MHC in the immunological synapse and therefore DNCB sensitization reflects the classical model of T cell antigen recognition (Pickard *et al.*, 2007). Here, we showed that clinical responses to DNCB were significantly attenuated in AD (Fig. 3), suggesting that individuals with AD show a significantly impaired immune response to this antigen. Furthermore, ADFM and ADWT showed similar impairments in cutaneous immunity to DNCB, suggesting that the immune dysregulation is not filaggrin dependent but is due to other AD-specific epidermal factors and may be protective against more inflammatory Th1 responses classically associated with allergic contact dermatitis (ACD). These findings are in line with clinical observations reporting a lack of association between *FLG* mutations with a phenotype of patients with both AD and ACD (Carlsen *et al.*, 2011). Nickel ACD has been reported to associate with the presence of *FLG* mutations (Thyssen *et al.*, 2008) but others have only found a non-significant trend towards this finding (Carlsen *et al.*, 2010). The lack of histidine-rich filaggrin proteins leading to increased penetration of the metal could explain a nickel-specific association with loss of filaggrin protein in *FLG* mutations but there is no evidence that other contact allergens bind filaggrin (Carlsen *et al.*, 2010). Also, *FLG* mutations impair impermeability of the epidermal water barrier, and because nickel is water soluble it is probable that percutaneous absorption of nickel will be greater in people with *FLG* mutations.

It has previously been shown that higher doses of DNCB correlate with increased sensitizing potential and therefore, it would be expected that skin penetration is highly efficient in all individuals at a dose which sensitizes 100% of the population as used here (Friedmann *et al.*, 1983). To enhance the detection of

differences in the penetration studies, we showed that a dose of DNCB which would be expected to sensitize approximately half the population, efficiently penetrated to the basement membrane in AD and controls. Although these data do not exclude the possibility of differences in DNCB penetration at lower concentrations, we feel that the equal DNCB penetration at the tested dose allowed us to directly compare the immune responses to DNCB in AD patients and healthy individuals.

Therefore, in this study we could be sure that priming of the immune response was initiated by equal cutaneous exposure to antigen in both AD and controls. DNCB would then be carried and presented by cutaneous dendritic cells for T cell priming in the draining lymph node. In this way, we hoped to determine the effect of the skin on induction of subsequent immune responses in AD and controls without significant effects manifested by differences in other immunological compartments. Although, variation in lymph node function could contribute to the observed differences, it has been previously shown that vaccination in the arm (bypassing the skin, utilizing axillary lymph node drainage as in this study) is equally effective at inducing Th1 responses in AD and controls (Leung *et al.*, 1988; Schneider *et al.*, 2010). Furthermore, we showed that in AD, the circulating lymphocyte compartment was not uniquely skewed towards Th2 (Fig. 2) as compared to controls and previous reports have shown that serum levels of IL-4 in AD are normal (Takahashi *et al.*, 1992). Despite the evident reduced skin responsiveness to DNCB in AD, both ADWT and ADFM made significantly greater DNCB-specific Th2 responses than controls (Fig. 5). Taken together, it seems likely that skewing of DNCB responses towards Th2 in AD is likely to be due to skin-specific regulation of DC signals determining polarization of immune responses to antigens encountered in the skin. We speculate that the altered function of cutaneous dendritic cells may explain the induction of DNCB-specific Th2 cells in AD which would result in a less inflammatory response on antigen challenge accounting for the impaired clinical responses seen here. We have also found that in biopsies from positive DNCB skin challenge sites, gene transcription of IL-10 is higher in AD than controls (data not shown) which may suggest that co-regulation of regulatory cytokines in skin is important in manifesting a reduced inflammatory reaction. Interestingly, controls were better able to maintain their Th1 memory population than AD patients (Fig. 5) which we further speculate may suggest that altered programming by keratinocyte

derived TSLP in AD skin results in preferential maintenance of memory Th2 populations at the expense of Th1 skin sensitized memory cells (Wang *et al.*, 2006).

These data would be predicted to be relevant for allergic contact dermatitis. Recent evidence suggests that the allergic contact dermatitis in AD is identified at least as frequently as in controls (Gittler *et al.*, 2012). Our data would suggest that once sensitized, individuals with AD are likely to elicit weaker responses on cutaneous re-exposure. This may suggest that the sensitivity of patch testing may be lower in AD, thus allergic sensitization may be higher than characterized by standard testing.

The altered innate immune responses of the epithelia and particularly the epidermis, suggest mechanisms by which adaptive immune responses activated via the trans-epidermal route can be programmed or directed towards Th2 responses. While there is deficient production of key components of anti-microbial innate defences, interferon-alpha in the bronchial epithelium and beta defensins and cathelicidins in the epidermis, there is augmented production of prostaglandin E2 (Bao *et al.*, 2011; Kalinski, 2012) GM-CSF (Willart *et al.*, 2012) and thymic stromal lymphopoietin (TSLP) (Soumelis *et al.*, 2002). These are all able to condition T cells towards the Th2 phenotype by modifying their activation by dendritic cells. Most particularly, TSLP can program dendritic cells via increased expression of OX40 to induce the generation of inflammatory Th2 cells characterized by the production of TNF- α in addition to the classical Th2 cytokines (Liu, 2006; Oyoshi *et al.*, 2010). We wished to test the hypothesis that the epidermis of individuals with atopic dermatitis does indeed programme a Th2 response to antigens encountered via the trans-epidermal route. We took the approach of inducing a new immune response with a widely used experimental organic contact sensitizer, 2,4-dinitrochlorobenzene (DNCB), not found in the environment. A major advantage of DNCB is that since it is not water soluble, its absorption into human epidermis would not be affected by the defective water-permeability barrier found in many atopic patients – which we confirm. The normal human response to this and most small molecular contact sensitizers is known to be mediated by Th1 and Tc1 T cells (Pickard *et al.*, 2007). We have been able to show that in complete contrast to the normal, non-atopic response, when individuals with AD are sensitized through normal skin with DNCB, they indeed generate a substantial Th2 response to this agent. This demonstrates that the skin

in humans with AD contributes to programming the adaptive T cell response towards the Th2 type, which is to our knowledge previously unreported. This finding should direct future research to focus on the role of epithelia of skin and airways in programming the adaptive immune responses that characterize the atopic state.

The data presented here demonstrate that AD skin is immunologically dysfunctional and that this manifests as impaired cutaneous reactivity to chemical antigens and is associated with Th2 polarization of the adaptive immune response. TSLP, a master regulator of Th2 polarization, has been shown to be up-regulated in lesional but not in non-lesional AD skin (Soumelis *et al.*, 2002), therefore as these data were from non-lesional AD, they suggest a different AD-specific Th2 pathway. AD skin-specific Th2 skewing is of relevance to the disease pathogenesis and characterization of key epidermal signals in AD skin critical to Th2 polarization will offer therapeutic targets for the T cell mediated allergic component of the disease and potentially for preventing future atopic disease in these individuals.

Materials and Methods

Subjects

Informed, written consent was obtained as per approval by the local research ethics committee (NRES07/Q1704/46) in adherence with the Helsinki guidelines. Adult AD patients with mild to severe disease (mean objective SCORAD 33.5 ± 11.7 , Supplementary Table 1) were recruited through the Dermatology Centre, Southampton University Hospital NHS Trust for comparison with a cohort of healthy non-atopic controls. All AD patients fulfilled the diagnostic criteria for AD as defined by the U.K. AD diagnostic criteria (Williams *et al.*, 1994a; Williams *et al.*, 1994b; Williams *et al.*, 1994c). Objective SCORAD was measured as described previously (Schmitt *et al.*, 2007).

Quantification of contact sensitization by DNCB

Individuals with AD and controls were sensitized to dinitrochlorobenzene (DNCB; Sigma, UK) at $60\mu\text{g}/\text{cm}^2$ in acetone by epicutaneous patch application to upper inner arm skin at a non-lesional site (free from eczema) according to our previous method (Pickard *et al.*, 2009; Pickard *et al.*, 2007). In all AD volunteers this site showed no evidence of active eczema and was not being treated with topical therapy. Four weeks later strength of sensitization was determined by measurement of responses to simultaneous patch challenge with doses of 6.25, 8.8, 12.5 and $17.7\mu\text{g}$ of DNCB to the opposite upper inner arm (free from eczema). Clinical responses were quantified 48 hours later at each challenge site by measuring the change in skin fold thickness (Harpender Skinfold calipers, UK) and erythema index (DiaStron, UK) as described previously (Paramasivan *et al.*, 2009; Patil *et al.*, 1998). Responses at challenged sites were expressed as net increase after subtraction of baseline readings taken from adjacent normal skin. Values at each challenge site were summed to give a single value approximating to the area under the curve (AUC).

T Cell Culture

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by centrifugation on Lymphoprep (Axis-Shield PoC, Oslo, Norway) washed, and resuspended in R10HS: RPMI medium (Invitrogen, Carlsbad, CA) supplemented with penicillin/100iu/ml, streptomycin $100\mu\text{g}/\text{ml}$, heat inactivated pooled human serum (Sigma, Poole, UK). DNCB was added at a final concentration of $2\mu\text{M}$. The cells

were incubated at 37°C with 5% CO₂ with IL-2 (100iu/ml) and IL-7 (1µg/ml) supplemented at day 7. At day 13 these cells were harvested, washed and returned overnight to wells containing R10HS..

ELISpot assays

Ex vivo PBMCs were tested at 2.5×10^5 per well; for T cell lines input cell numbers were 5×10^4 per well. DNCB was added at a final concentration of 2µM. For phytohaemagglutinin (PHA) and staphylococcal enterotoxin B (SEB). experiments the final concentrations are shown in the figure (Fig. 2). The plates were incubated overnight at 37°C in 5% CO₂ and were developed with streptavidin-alkaline phosphatase (Mabtech AB, Cincinnati, OH) and alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA). All experiments included vehicle as a negative control, and phytohemagglutinin (PHA) was used as a positive control. DNCB-specific responses were calculated by subtracting the counts from control wells, and the results were expressed as spot forming units (sfu) per million cells plated. Positive responses were recorded as those responses greater than the mean of all the background samples plus 3 times the standard deviation of the background population.

Intracellular cytokine staining

Culture expanded DNCB-specific lymphocytes were activated with SEB (0.1µg/ml) and anti-CD28 (1µg/ml), with GolgiPlug (BD Biosciences, UK). Cytofix/Cytoperm kit (BD Biosciences, UK) was used according to the manufacturers instructions. Flow cytometric analysis with FACS Aria flow cytometer (Becton Dickinson, USA) was undertaken following lymphocyte gating on Forward/Side scatter and exclusion of dead cells (AQUA, Invitrogen, UK). Subsequent gating on CD3-FITC and CD4-PerCP (BD Biosciences, UK) was based on appropriate negative controls to demonstrate IFN-γ and IL-4 (Invitrogen, UK and BD Biosciences, UK) positive cells for analysis with FlowJo software (Treestar, USA).

DNCB skin penetration and staining of frozen sections

Skin explants from mastectomy tissue (healthy controls), AD FLG wild-type, and AD FLG variant subjects were mounted in modified Franz diffusion chambers. Skin penetration studies were undertaken as described previously (Franz *et al.*, 2009; Gawkrödger *et al.*, 1989). Briefly, DNCB (20µg/cm²) in vehicle (acetone)

was applied to the epidermal surface at 33% of the clinically used sensitizing dose to increase the possibility of identifying small differences in penetration. Explants were incubated overnight at 37°C in 5%CO₂ and then snap frozen in liquid nitrogen. Frozen skin sections were cut using a cryostat and stained with a polyclonal goat anti-dinitrophenyl antibody (Sigma-Aldrich; 1:2000) for 1 hour at room temperature, then a FITC-labeled anti-goat secondary antibody was added (Sigma-Aldrich; 1:400). Staining was visualized with fluorescent or confocal microscopy and images were analyzed with ImageJ (Abramoff *et al.*, 2004). The investigator undertaking image analysis was blinded to the subject disease or *FLG* status.

FLG genotyping (see supplementary material for further information)

FLG mutation analysis was performed as described previously (Sandilands *et al.*, 2007) (Enomoto *et al.*, 2008).

Statistical Analysis (see supplementary material)

Conflict of Interest

None declared.

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Legends

Figure 1. DNCB penetration through epidermis

DNCB (20 μ g/cm²) was applied to the epidermis of skin biopsies from controls (a,b), atopic dermatitis without filaggrin mutation (ADWT; c,d) or filaggrin mutation heterozygote (ADFM; e,f) individuals. DNCB penetration was analyzed by immunohistochemistry with anti-DNCB (a-f) or representative antibody control (g) (green). Penetration of DNCB was assessed by measurement of the relative fluorescence of anti-DNCB (12 independent readings) from the base of the stratum corneum versus from the basal layer, as indicated by white dotted lines, using image analysis software (h). Scale bar = 10 μ m. Error bars represent mean $\pm$ SEM.

Figure 2. Circulating lymphocytes in AD show a normal Th1/2 ratio

16 hour IFN- γ ELISpot (a, b) and IL-4 ELISpot (c, d) assay of ex-vivo PBMC activation with either PHA (left panels) or SEB (right panels) in control individuals (grey lines) or AD (black lines). (e) Ex-vivo PBMC IL-4 ELISpot stimulated with House Dust Mite (HDM) extract. AD n=18; Controls n=10. *p<0.05.

Figure 3. AD subjects have impaired allergic contact dermatitis responses to DNCB.

Clinical response to epicutaneous challenge with DNCB 1 month following sensitization through the skin in AD (black lines) and controls (grey lines). Change in skinfold thickness (a) and erythema index (b). (c) Area under the curve (AUC) for change in skin fold thickness and (d) erythema index for DNCB-elicitation reactions in Controls or AD with (ADFM) or without filaggrin mutations (ADWT).

Figure 4. DNCB-specific Th1 and Th2 cells

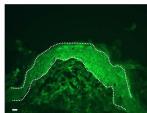
(a, d) DNCB-specific responses detected in PBMC (Ex vivo; grey bars) or after short term culture with DNCB (Line; black bars) by IFN- γ (a) or IL-4 (b) ELISpot from a DNCB-sensitized atopic individual. (b, c, e, f) Flow cytometric analysis of IFN- γ or IL-4 production by activated DNCB-expanded T cell lines (c, f) or unstimulated control PBMC (b, e). Low frequency Th1 (a, c) and Th2 (d, f) cells can be detected ex-vivo, but higher frequency responses are detected after short term culture expansion. Population frequency (%) as indicated in appropriate quartiles. Representative examples shown.

Figure 5. Sensitization through the skin in AD shows unique skewing towards antigen-specific Th2 responses.

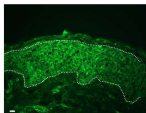
ELISpot assay of DNCB-specific (a) IL-4 or (b) IFN- γ production by PBMC from non-sensitized controls (horizontal striped bars) or DNCB sensitized controls without AD (Non-AD, black bars) or DNCB sensitised individuals with AD (grey bars) 4 weeks after cutaneous DNCB sensitization AD. (c) DNCB-specific IL-4 from DNCB sensitised controls or AD with (ADFM) or without filaggrin mutations (ADWT). AD n=16 (Supplementary table 1); Controls n=10. Error bars represent mean $\pm$ SEM. Long-lived DNCB-specific Th2 memory cells in AD shown by ELISpot assay of DNCB-specific (d) IL-4 or (e) IFN- γ production by PBMC from controls or individuals with AD 3 months after re-challenge with DNCB (4 months after sensitization). AD n=10; Sensitized controls n=10; Non-sensitized controls n=6. Error bars represent mean $\pm$ SEM. ns, no statistical significance.

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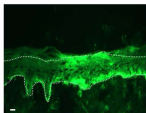
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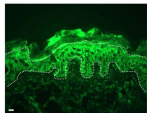
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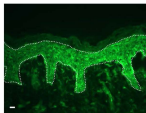
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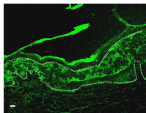
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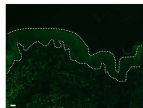
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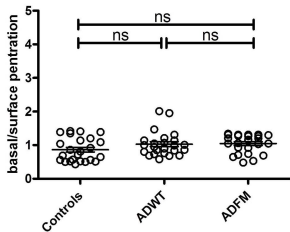
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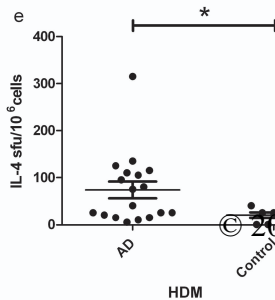
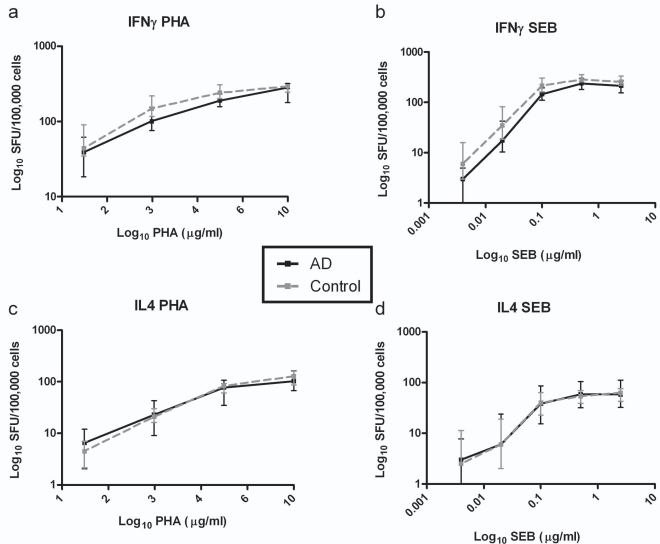


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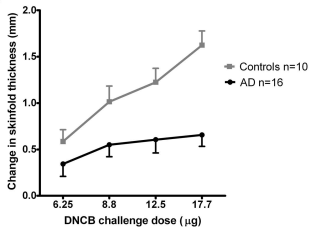


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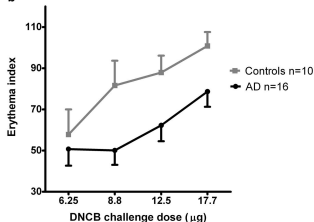




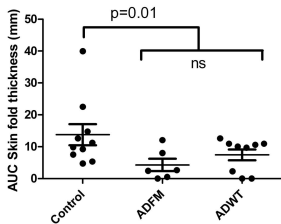
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