Comparative *in situ* topoproteome analysis reveals differences in patch test-induced eczema: cytotoxicity-dominated nickel versus pleiotrope pollen reaction

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Abstract: A subgroup of patients with atopic eczema develops acute eczematous reactions to type I allergy-inducing agents such as pollen that clinically resemble type IV allergies induced by haptens like metal ions. To clarify the underlying immunologic mechanisms, this study was designed to map the inflammatory *in situ* topoproteome of eczematous responses to grass/birch pollen and nickel by using atopy patch test (APT) and nickel patch test (NPT) as an appropriate clinical model, respectively. Biopsies from NPT ($n = 6$) and APT ($n = 6$) with positive reactions at 72 h were analysed by multiple epitope ligand cartography (MELC), which enabled to investigate coexpression of 49 different epitopes immunohistochemically in a single given tissue section. Colocalisation of IgE and FcεRI was investigated by confocal microscopy. Compared with APT responses, NPT reactions were dominated by cytotoxic TIA-1 + and CD8 + T cells. In contrast, the immune response in APT reactions appeared more pleiotrope – as detected by colocalisation analysis. Multiple combinatorial molecular phenotype (CMP) motifs containing naive, early maturation and memory T cell (CD45RA, CD7, CD44, CD45R0), and general activation markers (CLA, HLA-DR, CD13, CD29, CD58, CD71, CD138) were significantly higher expressed in APT when compared with NPT reactions. APT response was confirmed to be accompanied by IgE bound to FcεRI. In summary, our results demonstrate that the NPT reaction is clearly dominated by cytotoxic events, while the APT reaction to pollen grains is more heterogeneous and elicits a combined humoral and cellular immune reaction.

Key words: atopic dermatitis/eczema – atopy patch test – *in situ* topoproteome analysis – multiple epitope ligand cartography – nickel – pollen

Introduction

The pathogenesis of atopic eczema (AE) is based upon complex interactions between genetical predispositions and environmental factors (1). Clinical hallmark of AE is a chronic relapsing-remitting cutaneous inflammation that is caused by the combination of a disturbed epidermal barrier (2) with a consecutive high transepidermal water loss on the one, and a hyperreactive immune system on the other side. In this scenario, type I hyperreactivity reactions to air-borne or nutritious allergens are highly characteristic for AE patients. While the classical immediate hyperreactivity with clinical symptoms like rhinitis and eye swelling is well characterised and mediated by allergen-specific IgE, T helper (Th) 2 responses and mast cells, less is known about how these allergens influence severity of AE directly (3). The atopy patch test (APT) is a well-established model for the induction of acute eczematous reactions to type I allergy-inducing allergens in a subgroup of sensitised AE patients (4).

Both clinically and histologically, the APT reaction shares close similarities with a delayed type hypersensitivity (type IV) reaction to small molecules called haptens like nickel (allergic contact dermatitis, ACD) (5,6). Haptens penetrating the skin need to bind to self-proteins in order to get fully allergenic (7). The immune reaction against hapten/self-protein complexes is mediated by skin infiltrating cytotoxic CD8 + and CD4 + T cells that elicit apoptosis in hapten-presenting keratinocytes in an antigen-specific manner (8). Keratinocyte apoptosis and early loss of adhesion molecules like cadherins (9) are major events in the pathogenesis of ACD.

Although the general mechanisms of the immune responses underlying APT and nickel patch test (NPT) reactions are similar, several differences have been observed (6). While in general, APT reactions follow a faster kinetics, maximum flares of NPT reactions are stronger. Besides quantitative differences, several qualitative differences in the distribution of dendritic cell (6,10–12) and T-cell subsets (13–15) have been suggested.

Nevertheless, a detailed characterisation of leucocyte subtypes *in situ* remained largely obscure because of limits of classical immunohistochemistry. Taking benefit from the innovative technique '*in situ* topoproteome analysis' that enables to stain for up to 100 antibodies in the same section (16,17), the aim of our study was to describe differences in the leucocyte populations infiltrating APT and NPT reactions. Our data demonstrate that the NPT reaction is mediated by significantly higher amounts of cytotoxic CD8 + T cells, while APT immune responses are more complex – with significantly more activated naive and premature T cells, higher expression of CD1a and presence of receptor-bound IgE.

Materials and methods

Patients

Six AE patients and six patients with a positive NPT were enrolled into the study after written informed consent had been given. AE patients were diagnosed according to the criteria of Hanifin (18) and had sensitisations against grass and/or birch pollen (specific IgE class 3 or higher) and a positive APT. Allergen-specific and total IgE levels in serum were determined by CAP-FEIA (Pharmacia, Uppsala, Sweden). Patients with positive NPT ($n = 6$) had a typical clinical history, but negative personal and family history of AE or atopy. None of the patients had received systemic steroids or antihistamines or UV light on the test site at least 14 days prior to testing. The study followed the declaration of Helsinki protocols. The ethical committee of the Technical University of Munich approved the study.

Pollen and nickel patch test

Pollen patch tests were performed using the standardised procedure of APT (19) with 1 mg intact pollen grains instead of pollen extracts. Briefly, commercially available grass (*Phleum pratense* L.) and/or birch (*Betula alba* L.) pollen (Allergon, Ängelholm, Sweden) were applied in large Finn chambers (11 mm in diameter) on the back of the patients using petrolatum as a carrier. Petrolatum alone served as negative control. After 48 h, the Finn chambers were removed and the tested areas were marked. The test was evaluated after 48 and 72 h. Positive reactions were classified according to the European Task Force on Atopic Dermatitis (ETFAD) 2000 reading key (20). Epicutaneous provocation with nickel was performed using petrolatum containing 5% $NiSO_4$ (nickel[II]-sulphate 5%; Hermal, Reinbeck, Germany) applied on the patients back for 48 h. All reactions were classified using the criteria of the International Contact Dermatitis Research Group and ranged between 1 and 3, according to the state of inflammation.

Tissue processing

Punch biopsy specimens (5 mm in diameter) were taken under local anaesthesia (1% lidocaine) from the centre of the patch test areas 72 h after nickel/pollen application. Each patch test field was biopsied only once. Biopsies were immediately frozen in liquid nitrogen and stored at −70°C until further handling. Tissue sections of 4 μm thick were cut at a microtome.

Basic MELC set-up

Multiple epitope ligand cartography robot technology (16,17,21,22) was applied using a Toponome Imaging-Cycler (Fig. S1). Briefly, a slide with a skin section was positioned onto the stage of an inverted fluorescence microscope (Leica DM IRE2, Solms, Germany; 20 × air objective lens), equipped with fluorescence filters for Fluorescein isothiocyanate (FITC) and phycoerythrin. By a robotic process of on/off-pipetting, the specimen was incubated with fluorophore-labelled tags and wash solutions under temperature and time control. The phase contrast view and fluorescence signals were recorded by a cooled CCD camera (Apogee KX4, Apogee instruments, Roseville, CA, USA, 2048 × 2048 pixels), followed by soft bleaching. Recording of all image data and coordination of all system components were controlled by software from Meltec GmbH, Darmstadt, Germany and Co. KG. All these processes (tag incubation/fluorescence detection/soft bleaching) were part of a fully automated cycle repeated for any number of tag-binding sites (epitopes).

MELC tag (antibody) library and MELC image processing

A validated MELC library of 49 tags (mostly antibodies) was used. The computer platform of the MELC robot

stored the phase contrast and raw fluorescence images for all tag-binding sites (epitopes) and chosen visual fields. As described in detail earlier (17,21,22), image preprocessing mainly comprised (i) pixel-precise overlay of fluorescence images, (ii) image correction for background and illumination faults and (iii) exclusion of invalid pixels that were not part of the information associated with the biological specimen (e.g. section artifacts).

Binarisation was performed under expert supervision for the expression of each tag-binding site (epitope) in relation to corresponding positive and negative biological and technical controls. Overlay of binarised images formed a matrix of combinatorial molecular phenotypes (CMPs; Fig. 1) (17,21,22), which represented binary coded vectors for n tag-binding sites (epitopes) being expressed (='1') or being not expressed (='0') in relation to each pixel or topographic micro unit of a visual field (4 Mega pixels).

Quantification of CMP data from MELC analysis

The degree of expression of a given CMP was quantified in relation to a horizontal skin width standardised to 100 μm (in parallel to the epidermo-dermal basal membrane zone; Fig. S1). The corresponding dimension was 'pixel events normalised to horizontal skin width (100 μm) (21,22).

Data analysis focused mostly on total skin, i.e. the epidermo-dermal compartment. However, by restriction to appropriate 'background' CMPs, the following subcompartments could additionally be identified:

(1) epidermis (keratin+/CD138 +);

(2) dermal vessels (CD31 + /SMA+/CD34 + /epidermis-) and

(3) extravascular dermis (whole section without epidermis and vessels).

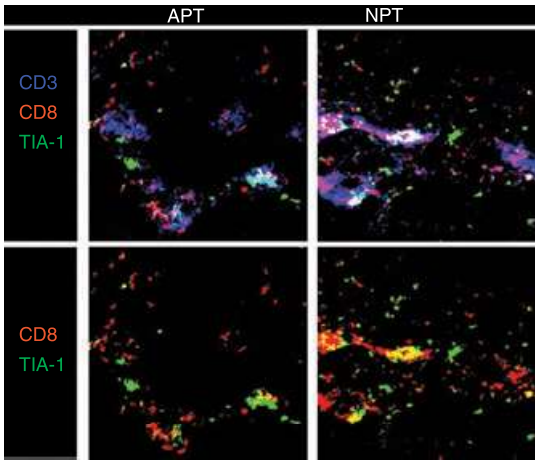

Figure 1. NPT reactions are dominated by cytotoxic and CD8 + T lymphocytes. Shown are expression and colocalisation of CD3, CD8 and TIA-1 in a postprocessing RGB imaging of one representative APT and NPT reaction.

Given that the *stratum corneum* is prone to unspecific antibody binding, it was excluded from all quantitative analysis so as not to bias the results.

Confocal microscopy

Frozen tissue sections were incubated with the primary antibodies (polyclonal rabbit anti-IgE, Genetex, San Antonio, Texas, USA; mouse anti-FcεRI, clone 22E7, kindly provided by Prof. Wollenberg, LMU Munich) overnight at 4°C in the dark. After extensive washing steps in Tris-buffer, secondary antibodies (polyclonal goat anti-rabbit Cy2, Genetex; polyclonal goat anti-mouse Cy3, Genetex) were added for 1 h and incubated in the dark. In the last 20 min, 300 nM 4′,6-Diamidino-2-phenylindol (DAPI) was added to counterstain nuclei. Following repetitive washing, slides were embedded in tissue medium (DAPCO, Dexter, Michigan, USA) and immediately investigated under a confocal microscope (Carl Zeiss, Jena, Germany). Analysis was performed using the software 'LSM510' (Zeiss).

Statistical analysis

Descriptive statistics were performed by unpaired and paired Student's t-test ($P < 0.05$). Multiple parameter testing were executed by Wilcoxon test ($P < 0.022$) and ANOVA with *post hoc* Scheffè, Bonferroni or Games–Howell test ($P < 0.05$, each). To increase the statistical power, we compared only fully separated cohorts of values. Statistical data analysis was run using SPSS software package, Stanford, Virginia, USA.

Results

A significantly higher number of total leucocytes, in particular cytotoxic CD8 + T cells, infiltrate the NPT reaction

To get a general impression on the quantity of the cellular infiltrate, we compared in a first step the total expression of single leucocyte and dendritic cell markers in NPT reactions to $NiSO_4$ ($n = 6$) with APT reactions to native pollen grains ($n = 6$).

Both total CD45 + leucocytes and the CD8 + T-cell subpopulation were significantly higher expressed in NPT (Table S1). Other T-cell markers like CD2, CD3 and CD4 also tended to be higher expressed, but the differences were not significant. Infiltrating T cells and, in particular, CD8 + cells showed a higher cytotoxic potential in NPT as they expressed significantly more TIA-1 (Fig. 1).

Leucocytes from NPT express higher numbers of anti-apoptotic Bcl-2

Alongside with their higher cytotoxic potential, also resistance to apoptosis was increased in NPT infiltrating leucocytes. The anti-apoptotic molecule Bcl-2 was significantly

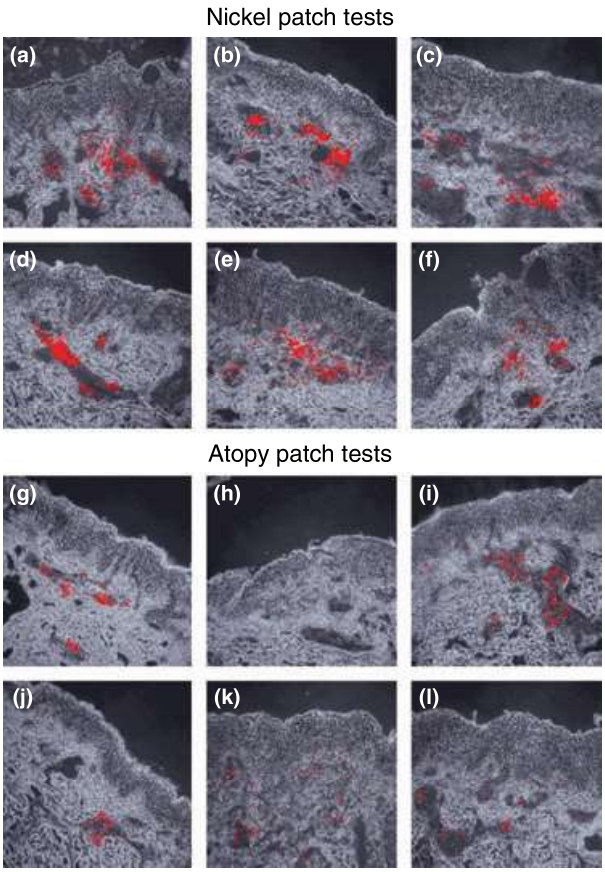

Figure 2. Lymphocytes derived from NPT reactions express higher levels of the antiapoptotic molecule Bcl-2. Combinatorial molecular phenotype motif CD45/Bcl-2 (motif number 2 in Table S2) is shown in NPT (a–f) and APT (g–l) lesions.

higher expressed both in single epitope expression analysis (Table S1) and in coexpression analysis with CD45 (Fig. 2).

In contrast to the number of infiltrating leucocytes, CD1a+ dendritic cell subpopulations showed a tendency to be higher expressed in APT reactions (Table S1). However, differences were not significant.

APT infiltrate contains significant higher numbers of activated naive and premature T cells as detected in coexpression analysis

In colocalisation analysis, naive and early maturation T-cell markers were significantly higher expressed together with standard T cell and/or activation markers in APT than in NPT lesions (Table S2; Fig. 3). Among these were motifs containing the naive T-cell marker CD45RA (HLA-DR/HLA-DQ/CD13/CD45RA; CLA/CD13/CD45RA) and the premature and early maturation T-cell markers CD7 (CD7/CD138; CD7/CD29/CD138; CD7/CD58) and CD44 (CLA/CD44; HLA-DQ/HLA-DR/CD13/CD44; CLA/CD44/CD45; HLA-DQ/CD2/CD13/CD44; CLA/CD2/CD44; CLA/CD3/CD44; CLA/CD3/CD44/CD45).

Infiltrating and resident cells express higher numbers of migration, adhesion and general activation markers in APT

Although the overall expression of the single markers did not differ significantly, colocalisation analysis of adhesion molecules and general activation markers such as CD13, CD29, CD58, CD71 and MHC class II molecules revealed that these markers were significantly higher expressed together with leucocyte (CD45), T cell (CD3, CD45RO) and macrophage (CD68) markers in the APT reaction. The following CMP motifs were significantly higher expressed in APT: HLA-DQ/HLA-DR/CD13/CD45, CLA/CD3/CD13, CLA/CD13/CD45RO, CLA/CD13/CD38/CD45RO, HLA/CD13/CD71, CLA/CD13/CD71, CD13/CD68, CD34/CD68, CD29/CD34/CD68, CD29/CD58/CD68/CD71, CD58/CD68/CD71 (Table S2).

In line with the general up-regulation of adhesion and activation molecules of infiltrating immune cells, also resident cells showed a significantly higher activation status in the APT reaction. CMP with a statistically significant higher expression in APT when compared with NPT reactions were CD29/CD34, CD13/HLA-DQ/CD71 and HLA-DR/HLA-DQ (Table S2).

In contrast, the costimulatory molecule CD26 was significantly increased in the epidermal compartment of NPT reactions.

APT reaction is accompanied by abundant receptor-bound IgE

To investigate the role of humoral immune factors in the APT reaction, we analysed the expression of IgE using confocal microscopy. In line with previous reports, we confirmed that IgE was abundantly present 72 h after pollen application. IgE was bound almost exclusively to the high-affinity IgE receptor (FcεRI), free IgE was not observed at this time point of the reaction (Fig. 4). In NPT responses, IgE was not detectable (data not shown).

Discussion

T cellular immune responses in the skin of AE patients are crucial in the pathogenesis of this skin disease. Here, we provide a detailed *snap shot* analysis of the *in situ* morphology of APT reactions to pollen grains in comparison with acute ACD responses to nickel. Our data demonstrate that NPT reactions are clearly dominated by cytotoxic T cells, while APT reactions to pollen are more complex and heterogeneous – with infiltration of various naive and premature T cells, high expression of general activation markers and receptor-bound IgE in the skin.

The detailed analysis in this study permits gaining insights into the underlying immune reactions to nickel and pollen. The NPT is a straight-lined reaction, where nickel

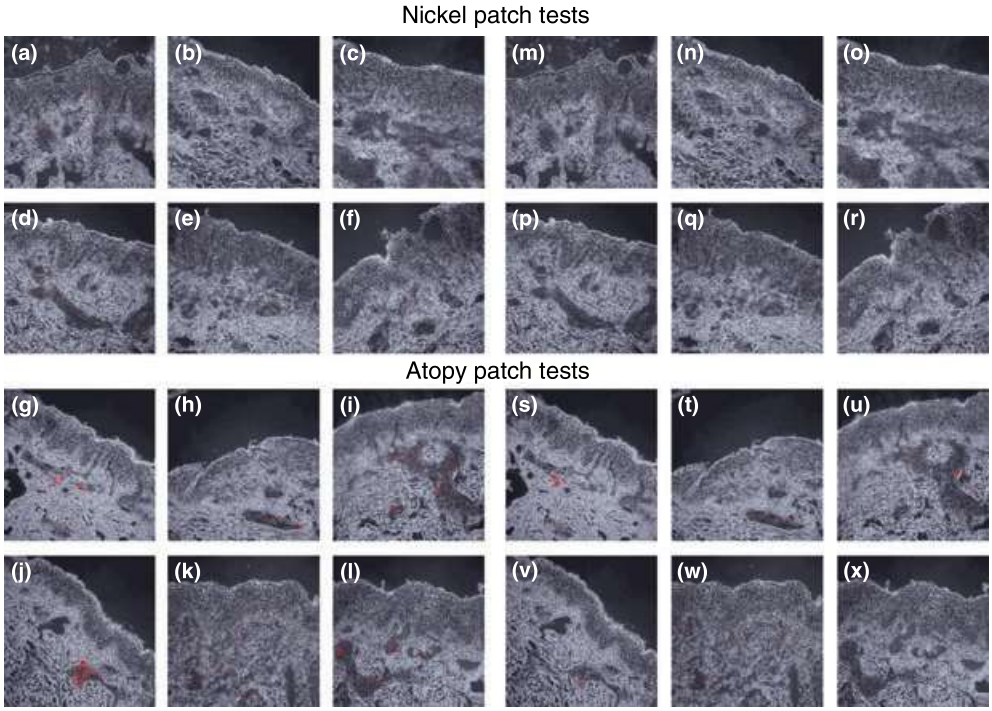

Figure 3. Naive and premature T-cell markers are significantly higher expressed in APT lesions. Combinatorial molecular phenotype motifs CLA/CD44/CD45 and HLA-DR/HLA-DQ/CD13/CD45RA (motifs number 11 and 38 in Table S2) are shown in NPT (a–f and m–r) and APT (g–l and s–x) lesions (n = 6 each).

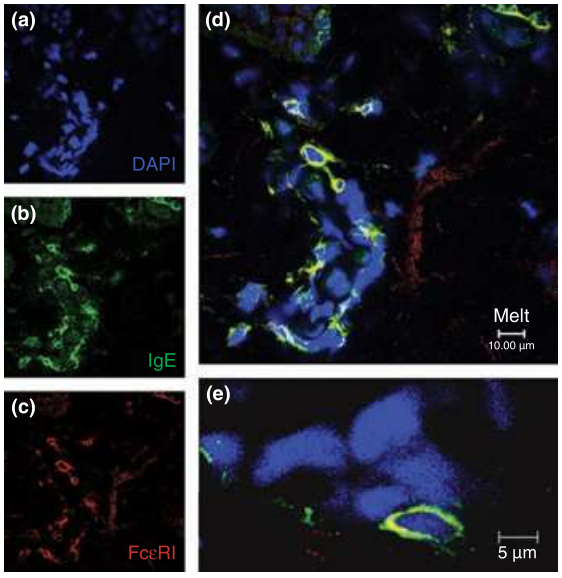

Figure 4. APT reaction is accompanied by receptor-bound IgE. Shown is one representative staining of an APT lesion with IgE (a), the high-affinity Fcε receptor I (b), and counterstaining with nuclear dye (DAPI, c). (d, e) Overlay.

binds to surface proteins of resident cells and APC, cytotoxic CD8 + TIA-1 + T cells are recruited into the skin and induce apoptosis in keratinocytes (8). The higher cytotoxic potential of lymphocytes correlates with higher expression of the anti-apoptotic and activation-dependent protein Bcl-2 (23). NPT reactions are dominated by type I cytokine producing T cells (6,24) and the reaction is self-limited when the triggering factor nickel is removed.

In contrast, immune responses to pollen in AE patients are more heterogeneous and complex. Although the overall quantity of T-cell markers is lower than in reactions to nickel, different populations of premature T cells (CD45RA+, CD7 + [25], CD44 + [26]) are significantly higher expressed in colocalisation with markers of (activated) T cells in APT responses. In addition, adhesion molecules and general activation markers are significantly higher expressed in colocalisation with T cells and macrophages in APT reactions. Among these are CLA, a major skin-homing antigen that is further induced in the skin upon activation (25); CD13 (myeloid associated antigen) that enhances monocyte trafficking and is regarded to be a general activation marker of the immune system; CD29 (integrin β1) that has been suggested as a marker to distinguish APT from NPT lesions previously (26); the CD2-binding antigen CD58 necessary for T-cell adhesion and stimulation by APC (27); the early activation marker CD71 (transferrin receptor) previously reported to be increased in CLA+ T cells in AE (28); the adhesion molecule CD138 (Syndecan) that is preferentially expressed in

epithelial cells (29) and plasma cells (30); and the MHC class II molecules HLA-DR and HLA-DQ that are regarded as general immune activation markers and are up-regulated in keratinocytes after stimulation (31). In parallel to infiltrating leucocytes, skin resident cells like CD34 + endothelial cells show a significantly higher expression of leucocyte adhesion molecules. Finally, IgE bound to the high-affinity receptor FcεRI is detected only in APT responses.

Two different determinants might be discussed to explain the higher complexity of the APT immune reaction when compared with nickel induced eczema: first, the nature of the allergen itself and, second, the general pathogenetical characteristics of AE. Most likely, both determinants are phenomenologically contributing to the observed differences.

As for the first and in contrast to nickel, pollen grains contain a high number of protein allergens (32), but possibly also lipid antigens that are presented via CD1 molecules (33). Thus, pollen grains are more than just protein allergen carriers (34,35). In addition, pollen grains release pro-inflammatory lipid mediators upon contact with the skin (36). These so called pollen-associated active lipid mediators (PALMs) possibly contribute to the faster kinetic of the APT when compared with NPT reactions. Furthermore, PALMs promote a type 2 reaction (37) involving allergen-specific IgE that is highly characteristic for the early phase of the APT reaction.

Besides these antigen-based explanations, disease-specific mechanisms seem to contribute likely to the observed differences. In contrast to otherwise healthy patients with a positive NPT that were chosen in this study, several immune response pathways are deregulated in AE patients. First, T cellular immune response to allergens is Th2-biased in AE patients, probably because of increased Th1 apoptosis (38). Furthermore and in contrast to healthy individuals, 90% of AE patients suffer from skin-colonisation with microorganisms like *Malassezia* or *Staphylococcus aureus* (39). During APT reactions, the epidermal barrier gets disrupted and most likely skin infiltrating T cells come in contact with microbial-derived products such as allergens (40,41) and/or exotoxins (so called superantigens) (42) that further increase and alter T-cell immune responses towards an IL-17/IFN-γ and IL-18 mediated reaction (43–45). In addition to immune responses to microbial-derived products, an immune reaction against self-proteins like manganese superoxide dismutase is reported to contribute to the pathogenesis of AE (46,47).

Taken together, the observed broad immune response in APT reactions forms the basis for multiple inflammatory cascades beyond mere antigen recognition and therefore has direct consequences regarding treatment and outcome of AE. Namely, the infiltration of premature T cells raises the possibility that co-sensitisations could occur in the skin. The fact that a higher multiplicity of allergies is commonly observed in patients with AE (1), but rarely in those with nickel allergy (48), strengthens this concept. These observations are strong arguments for a rapid and consequent anti-inflammatory therapy of early AE skin lesions.

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Conflict of interest

Prof. Dr B. Bonnekoh and Prof. Dr H. Gollnick are scientific co-founders and scientific advisers of SkinSysTec GmbH, Magdeburg.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Skin tissue-related application platform of MELC robot technology. The MELC core process of repeated cycling of antibody/tag incubation, fluorescence imaging and photo bleaching provides a series of fluorescence images (left image stack of the flow diagram). The process may be continued up to a maximum marker number of at least n = 100. A basic mode of postprocessing involves r(ed)/g(reen)/b(lue) imaging directed to three selected, correspondingly coloured markers (left part of the illustration). An advanced mode of postprocessing comprises binarization of fluorescence images under i) expert supervision and ii) consideration of anatomical horizontal orientation of the basal membrane zone (right image stack). This is the precondition for analysis of the expression of single epitopes or combinatorial molecular phenotypes (CMP) in a given dimension (i.e. pixel events normalized, PEN, in relation to 100 μm horizontal skin width). This allows a subsequent rigorous statistical comparison of topo-proteome information in appropriate cohorts by means of a so-called topo-miner strategy (right part of the illustration).

Table S1. Single epitope expression in APT and NPT lesions. PEN (pixel events normalised in relation to 100 μm horizontal skin width) as a mean of APT ($n = 6$) and NPT ($n = 6$) patients ± SD and the p-value. EfaBS = Efalizumab Binding Site

Table S2. Combinatorial molecular phenotype (CMP) motifs with significant differences between APT and NPT lesions. Relative expression in PEN (pixel events normalised, mean ± SD for $n = 6$ patients with APT and NPT each).

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