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XXV Ciclo

Expression of IL-1 family members in human allergic contact dermatitis

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1. INTRODUCTION

1.1. Allergic contact dermatitis

Allergic contact dermatitis (ACD) is a common inflammatory skin disease in industrialized countries with a great socio-economic impact. It is one of the most common occupational diseases. In Europe about 20% of the general population suffers from contact allergy to at least one contact allergen. Most common are allergies to nickel, fragrances and preservatives. Allergic reactions to chromate and p-phenylenediamine (PPD) are generally less common but occur frequently in occupationally exposed subgroups of the population (Lunder *et al.*, 2000). Risk factors for allergic contact dermatitis (ACD) can be subdivided into acquired and inherent. Acquired risk factors are generally inflammatory skin diseases such as irritant contact dermatitis (ICD) and possibly atopic dermatitis, while inherent risk factors are genetic variances resulting in a higher susceptibility.

It is initiated when allergens, usually haptens, penetrate the skin and modify the host's structural proteins. The contact allergen activates dendritic cells (DCs) in the skin via 'pattern recognition receptors' such as TLRs. Subsequently naïve T helper (Th) cells are polarised upon specific recognition of the haptenated allergen by the major histocompatibility complex (MHC), co-stimulatory signals and cytokines such as IL-12, IL-4, IL-1b and IL-6; polarized cells are conveyed to the draining lymph nodes where they undergo activation and clonal expansion (Gober *et al.*, 2008). The lymphocytes thus activated then migrate to the peripheral tissue ready for subsequent encounter with the same antigen. In the elicitation phase, following re-exposure of the skin to the same contact allergen, hapten-specific cytotoxic CD8+ T lymphocytes (CTLs) release inflammatory cytokines and induce disease-specific local symptoms such as redness, papules and vesicles (Gober *et al.*, 2008) (Figure 1).

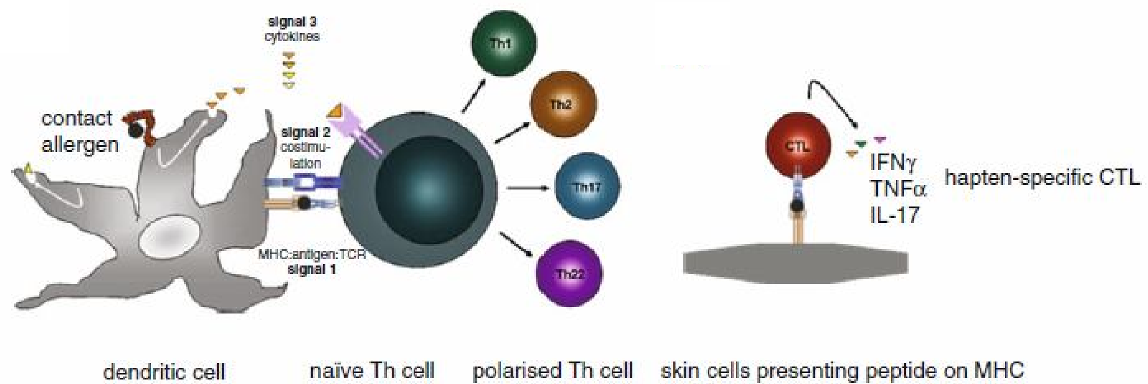

Figure 1. Lymphocyte-mediated immune mechanisms in contact allergy.

Thus, during the rapidly triggered innate immune response to contact allergens, among others, keratinocytes, mast cells and DCs are activated, and NK cells and neutrophils are recruited. Mast cells increase vascular permeability, thereby facilitating the recruitment of innate inflammatory cells. It has also been shown that mast cells contribute to the recruitment of leukocytes via TNF-α and support skin DCs maturation, migration and polarization of T-cell responses to IL-17 and IFN-γ production (Biedermann *et al.*, 2000, Kneilling *et al.*, 2009 Cumberbatch *et al.*, 201 Dudeck *et al.*, 2011).

1.2. The Interleukin-1 family

Interleukin-1 (IL-1), which as its name suggests was the first interleukin to be described, possesses many biological activities. Indeed, before its molecular identification in the mid 1980s, IL-1 had actually been studied for many years under various names (such as leukocyte endogenous mediator, haematopoietin 1, endogenous pyrogen, catabolin and osteoclast activating factor) that describe the multiple and varied biological functions of this cytokine. Over the past decade, several other members of the IL-1 family have been identified, and their important roles in innate and adaptive immune responses have been revealed. This family comprises 11 members with both pro- and anti-inflammatory activities. In 2009, a dramatic increase was observed in the number of publications on the function and potential disease involvement of the IL-1 family members. (Sims *et al.*, 2010)

IL-1α (also termed IL-1F1) and IL-1β (IL-1F2) bind both to IL-1RI, whereas IL-1Ra (IL-1F3) has an antagonistic effect on this receptor. These cytokines play an important role in immune regulation and inflammatory processes by inducing expression of many effector proteins, e.g. cytokines/chemokines (Dinarello, 2011). Excessive and/or dysregulated activity of these mediators is associated with tissue destruction and therefore the synthesis, secretion and biological activity of IL-1 cytokines have been identified as therapeutic targets for common inflammatory disorders such as rheumatoid arthritis (RA) (Burger *et al.*, 2006).

IL-18 (IL-1F4) binds to IL-18Rα with a pro-inflammatory activity. IL-18 is important in both innate and acquired immune responses; it stimulates neutrophil migration and activation as well as T helper 1 (Th1) cell differentiation and interferon (IFN)-γ secretion in a variety of cell types. IL-18 has a role in destructive inflammatory disorders (Gracie *et al.*, 2003) and is a potential therapeutic target although, currently, anti-IL-18 therapies are only at the preclinical trial stage (Anderson *et al.*, 2006).

IL-36α, IL-36β and IL-36γ (originally named IL-1F6, IL-1F8 and IL-1F9, respectively) bind to the IL-36 receptor (IL-36R, also named IL-1Rp2 or IL-1RL2) and a pro-inflammatory signal is initiated, whereas IL-36Ra, also termed IL-1F5, acts as the natural-occurring receptor antagonist. Similar to the classical IL-1 cytokines, IL-36α, IL-36β and IL-36γ are also involved in the initiation or regulation of immune responses (Sims *et al.*, 2010). Differently from IL-1α and IL-1β, IL-36α, IL-36β, IL-36γ and IL-36Ra are expressed in a restricted manner, primarily in the skin and other epithelial tissues, whereas the receptors are more widely expressed (Sims *et al.*, 2010).

IL-1F7 acts as an attenuator of IL-18 activity (Pan et al., 2001) and may act as an inhibitor of innate immune signaling.

IL-1F10 has been reported to bind soluble IL-1R1 to unknown effect. It has been shown to be expressed in the basal epithelium of skin and by proliferating B cells in tonsil germinal centres (Lin *et al.*, 2001). The genomic structure and amino acid sequence of IL-1F10 both indicate a

closer relationship to IL-1Ra and IL-36Ra than to the rest of the family, suggesting it may have an antagonistic role.

IL-1F11 is also known as IL-33; it binds to ST2 receptor and a pro-inflammatory activity is started. From studies using soluble forms of ST2 or antibodies to ST2, we know that IL-33 drives T helper type 2 responses (Schmitz *et al.*, 2005).

1.3. The IL-1 family and immune-mediated diseases

The key role of IL-1 family members in driving T cell, B cell and innate immune cell responses inevitably makes them important contributors to immune mediated diseases.

IL-18 is implicated in the pathogenesis of arthritis. Excessive production of IL-18 is found in both blood and inflamed joints of patients with rheumatoid arthritis. moreover, therapeutic inhibition of IL-18 alleviates disease in animal models of arthritis, often coincident with reductions in synovial IL-1 and TNF-α levels (Gabay *et al.*, 2009). However, the result of effective inhibition of human IL-18 in patients with rheumatoid arthritis has not been reported.

Conversely, inhibition of IL-1 not only stops further disease progression but also reverses established disease and is more effective than inhibition of TNF-α (Joosten *et al.*, 1999). However, the therapeutic use of the recombinant form of IL-1Ra, Anakinra (Kineret; Amgen/Biovitrum), in rheumatoid arthritis has yielded disappointing results, with fewer patients benefiting and higher disease scores than inhibitors of TNF-α or biologics (Mertens *et al.*, 2009).

Both IL-1α and IL-1β expression are enhanced in the inflamed intestinal mucosa of patients with inflammatory bowel disease, whereas IL-1Ra is only modestly increased, resulting in a significant decrease in the ratio of IL-1Ra to IL-1 (Casini-Raggi *et al.*, 1995). Inhibition of IL-1 is beneficial in several models of inflammatory bowel disease. The mechanism by which low levels of IL-1 secretion contributes to the pathology of Crohn's disease has not been identified but could relate to the hypothesis that the disease is in part caused by a dysfunctional mucosal

innate immune system, resulting in inadequate initial responses to infection or tissue damage (Yamamoto-Furusho *et al*., 2006).

Asthma is a syndrome of reduced airway flow and bronchial remodelling that is driven in part by unregulated and excessive pulmonary inflammation. The expression of IL-1β by macrophages in the alveoli and airway submucosa is increased in patients with asthma. In mouse models of airway inflammation provoked by either a protein (ovalbumin) or a small molecule (toluene diisocyanate) allergen, both IL-1α and IL-1β are required for full development of disease, including eosinophil infiltration into the lung, goblet cell hyperplasia, adhesion molecule expression, production of IL-4, IL-5 and allergen-specific IgG and Ige antibodies, and airway hyperresponsiveness (AHR). IL-1Ra administered at the allergen challenge phase reduces inflammation and AHR in response to ovalbumin (Wang *et al*., 2006). Moreover, although IL-18 is thought to be a Th1-type cytokine, multiple lines of evidence suggest it is a contributing factor in asthma.

IL-1 family members are abundantly expressed in the skin. Psoriasis is a common disease characterized by abnormal epidermal differentiation that leads to redness and scaling of the skin. It is thought to be driven by Th-1 and Th-17 cells, although there is also an epidermal component (Lowes *et al*., 2007). IL-1 family members are increased in the skin lesions of patients with psoriasis (Naik *et al*., 1999, Zhou *et al*., 2003, Blumberg *et al*., 2007), and in particular their expression can be induced by pro-inflammatory stimuli and suppressed by anti-inflammatory Retinoic acid and vitamin D (Balato *et al*., 2013). In particular, a recent study demonstrated that IL-36Ra, IL-36α, IL-36β and IL-36γ are able to drive antimicrobial peptides expression in skin and generate a psoriasis-like gene expression profile (Jhonston *et al*., 2011). In addition, these cytokines are not only effector cytokines downstream of their induction by Th-17 cytokines in the skin, but also inducers of many pro-inflammatory mediators that regulate cellular and soluble components of the local inflammatory milieu (Carrier *et al*., 2011).

1.4. IL-1 family and allergic contact dermatitis

Serveral cytokines, including members of the IL-1 family, play a fundamental role in the sensitization, elicitation and resolution phases of experimental contact hypersensitivity reactions (Kawase *et al.*, 2003, Antonopoulos *et al.*, 2008, Christensen *et al.*, 2012).

Langerhans cells produce IL-1β and IL-18 following activation by allergens. IL-1β and IL-18 upregulate the expression of co-stimulatory molecules by Langerhans cells and enable their migration out of the skin to the draining lymph node, where they can present antigen effectively to T cells. IL-18 also promotes contact hypersensitivity, and inhibition of IL-18 in rodents alleviates ongoing dermatitis (Plitz *et al.*, 2003). IL-1 and IL-18 contribute to T cell activation and recruitment to skin lesions (Wang *et al.*, 2002). Many contact sensitizers can activate the inflammasome in keratinocytes, leading to the production of both IL-1 and IL-18, and contact hypersensitivity reactions are defective in mice that lack any of several inflammasome components (Watanabe *et al.*, 2007).

IL-1α and IL-33 are known to be involved in contact hypersensitivity (Komai-Koma *et al.*, 2011, Nakae *et al.*, 2001). IL-1 is involved in the early inflammatory events, occurring within hours after sensitization, which dictate the development and severity of ACD (Bonneville *et al.*, 2007). Genetic susceptibility contributes to differences in host sensitivity to allergens and patients who have IL-1Ra gene polymorphism are prone to less effective antagonism to pro-inflammatory cytokine IL-1 (Ertam *et al.*, 2009). In addition, IL-18 has been shown to be a mediator of ACD both in human and murine models (Gangemi *et al.*, 2003, Corsini *et al.*, 2009, Zhang *et al.*, 2010).

IL-33 may play a role in the activation of these B1 B cells in ACD (Komai-Koma *et al.*, 2011). These cells are required especially in the early phase of the elicitation of ACD, by producing IgM which induces T-cell recruitment. IL-33 also activates mast cells and plays a role in neutrophil recruitment (Hueber *et al.*, 2011), thereby providing a further link between the different immune players.

The possible role of new IL-1 family members in the skin of patients with ACD is poorly understood. To better define the role of this family in skin inflammation, we obtained biopsies of normal donors, uninvolved and involved ACD skin and assessed the expression of IL-1β, IL-1Ra, IL-36s and IL-33 by real-time PCR, which we extended by immunohistochemical evaluation.

2. MATERIALS AND METHODS

2.1. Human subjects

The study population consisted of 25 subjects (mean age: 45.3 ± 2.7 years) with at least one positive reaction to patch test (Table 1). The intensity of reactions was evaluated according to the guidelines of the Italian Society of Allergic Dermatology (SIDAPA) in line with the recommendations of the International Contact Dermatitis Group (Fregert, 1981). Results were recorded as negative, doubtful, weakly positive (1+), moderate reaction (2+) and strong reaction (3+). The experimental protocols were approved by the Ethics Committee of the University of Naples Federico II and conformed to the principles outlined in the Declaration of Helsinki. Each subject gave written informed consent before entering the study.

2.2. *In vivo* expression of IL-1 family members in ACD

Skin biopsies (3 mm each) from involved and uninvolved sites (clinically normal skin) were taken from subjects with at least one positive reaction to patch test, and used for *in vivo* mRNA examination through real-time PCR. Skin specimens from normal donors (control) who had undergone plastic surgery and without a history of contact sensitization served as controls.

2.3. Immunohistochemical detection of IL-1 family members in ACD

The immunohistochemical detection of IL-1 family members was carried out on the skin biopsies from the eczematous lesion of ACD patients and from controls. Skin samples were immediately placed in tissue freezing medium (Jung, Leica, Wetzlar, Germany) and stored at −80° C. Five micrometer sections were cut with a cryostat and fixed with cold acetone for 10 min. The Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was used as follows: sections were incubated with blocking solution (horse serum diluted in buffer: phosphate buffered saline [PBS] + bovine serum albumin 1%) for 20 min at 22° C. Biopsies were stained with mouse anti-human IL-33 (0.5 µg/ml, clone Nessy-1, Alexis Biochemicals, San Diego, CA), IL-36Ra (5 µg/ml R&D System Inc., Minneapolis, MN), IL-36β (5 µg/ml R&D System Inc.) or goat anti-human IL-36α (15 µg/ml R&D System Inc.), IL-36γ (15 µg/ml R&D System Inc.) and

incubated overnight at 4° C. The sections were then washed in buffer and incubated with biotinylated secondary antibody for 30 min at room temperature. Peroxydase activity was revealed using DAB substrate (ImmPACT DAB, Burlingame, CA). Counterstaining was performed with hematoxylin.

2.4. *Ex vivo* expression of IL-1 family members in ACD

Skin biopsies (3 mm, each) from uninvolved skin of subjects affected by ACD were obtained for *ex vivo* assays. Skin specimens obtained from normal donors were used as controls. Biopsies were cultured as follows: a hole was punched in a transwell filter (pore size 1 µm; Beckton Dickinson Labware, Franklin Lakes, NJ). The biopsy was inserted into the hole, and the filter containing the biopsy was placed in a 12-well culture plate (Beckton Dickinson Labware) containing 1 ml of Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) containing 10% FBS (GIBCO), 2 mM L-glutamine (GIBCO) and antibiotics (100 IU/ml penicillin G, 100 µg/ml streptomycin, GIBCO) (Trowell, 1954). In this system the epidermis faces upwards at the liquid-air interface whereas the dermis is suspended in the culture medium. The tissue was incubated at 37° C in a humidified atmosphere containing 5% CO_2. Cultured uninvolved skin biopsies were exposed to the allergens that had previously elicited the positive patch test reaction and the vehicle only (petrolatum) as control. The same allergens were applied to cultured normal skin specimens and left in contact for 72 h at 37° C. The quantity of allergens used for these assays (30 mg) was the same used for the patch tests.

2.5. *Ex vivo* exposure to the irritant sodium dodecyl sulphate

Skin biopsies (3 mm, each) obtained from normal donors were used for irritation assays and exposed to the irritant compound sodium dodecyl sulphate (SDS) and to vehicle (H_2O). The irritation assay was performed as follows: IQ chamber filter paper discs (Chemotechnique diagnostics, Velling, Sweden), impregnated with 25 µl of non-cytotoxic concentrations of SDS (2 mM and 4 mM) (Sigma Chemical Co., St. Louis, MO) and vehicle (H_2O), were applied topically to the upwards surface of cultured skin for 72 h at 37° C (Ouwehand *et al.*, 2010).

2.6. *Ex vivo* anti-inflammatory activity of IL-36Ra

Skin biopsies (3 mm, each) from uninvolved skin of subjects affected by ACD were obtained in order to test IL-36Ra anti-inflammatory activity in *ex vivo* assays. Biopsies were cultured and treated with the corresponding sensitizing allergens as described above. Fifty microliters of recombinant IL-36Ra (rIL-36Ra; 1.25 µg/mL, R&D System, Inc.) were injected into the specimens and vehicle only (PBS) was injected into the remaining tissues as controls.

2.7. Nickel stimulation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from adult healthy volunteers with no history of contact allergic reactions and from patients with nickel contact allergy by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). They were then cultured in Roswell Park Memorial Institute medium (RPMI, GIBCO), supplemented with antibiotics (100 IU/ml penicillin G, 100 µg/ml streptomycin, GIBCO) and 10% fetal bovine serum (FBS, GIBCO), at a concentration of 2 x10^6 in 12-well plates. Cells were stimulated with $NiSO_4$ (10^{-5} M) and incubated at 5% CO_2 at 37° C.

2.8. Immunohistochemical detection of IL-33 after exposure to sodium dodecyl sulphate

Skin specimens obtained from 5 normal donors who had undergone plastic surgery and with no history of contact sensitization were exposed to the irritant compound SDS and used for IL-33 immunohistochemical analysis that was performed as described above.

2.9. *Ex vivo* activity of IL-33

Skin biopsies from uninvolved skin of 5 subjects affected by ACD, were obtained in order to test IL-33 activity through *ex vivo* assays. Biopsies were cultured and treated with the corresponding sensitizing allergens as described previously. Fifty microliters of anti-IL-33 polyclonal antibody (0.2 µg/mL, Abcam, Cambridge, UK) were injected into the specimens, whereas the vehicle only (PBS) was injected into the remaining tissues as controls.

2.10. RNA extraction, cDNA synthesis and real-time polymerase chain reaction

RNA was extracted from skin biopsy specimens (RNeasy Mini Protocol Qiagen) and cDNA was prepared (Transcriptor High Fidelity cDNA Synthesis, Roche, Indianapolis, IN) according to the manufacturer's instructions. PCR (LightCycler, Roche, Indianapolis, IN) was used to analyze the levels of expression of 18S, IL-1β, IL-1Ra, IL-36Ra, IL-36α, IL-36β, IL-36γ, IL-33, IL-8 and monocyte chemoattractant protein-1 (MCP-1/CCL2). PCR primers for the selected genes were designed based on published sequences, and their specificity was verified with BLAST alignment search. To confirm amplification of the expected size fragment, amplification products were characterized by agarose gel electrophoresis. Melting curve analysis was carried out after completion to confirm the presence of single amplified species. Relative mRNA expression levels were normalized to the expression of 18S.

2.11. Statistical analyses

Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc, La Jolla, CA). Student's t test was used to calculate statistical differences. Values of $p<0.05$ were considered significant.

3. RESULTS

3.1. *In vivo* expression of IL-1 family members in ACD

Given the pro-inflammatory nature of IL-1α and IL-1β, the identification of new members of the IL-1 family raised intriguing possibilities about their involvement in skin inflammation. Thus, we examined the constitutive expression of IL-1 family members using PCR in the sites of a positive patch test reaction (Involved), uninvolved skin (Uninvolved) of ACD patients, and in skin from normal donors (control) (Fig. 2). The expression of IL-1β (p<0.01) IL-1Ra (p<0.01), IL-36α (p<0.001), IL-36β (p<0.01), IL-36γ (p<0.01) and IL-33 (p<0.001) was significantly higher in involved skin of ACD patients than in skin from normal donors. Similarly, the expression of IL-1Ra (p<0.01) and IL-36γ (p<0.01) was significantly higher in uninvolved ACD skin than in control skin. By contrast, the expression of anti-inflammatory IL-36Ra cytokine in involved and uninvolved skin did not differ from that in control skin.

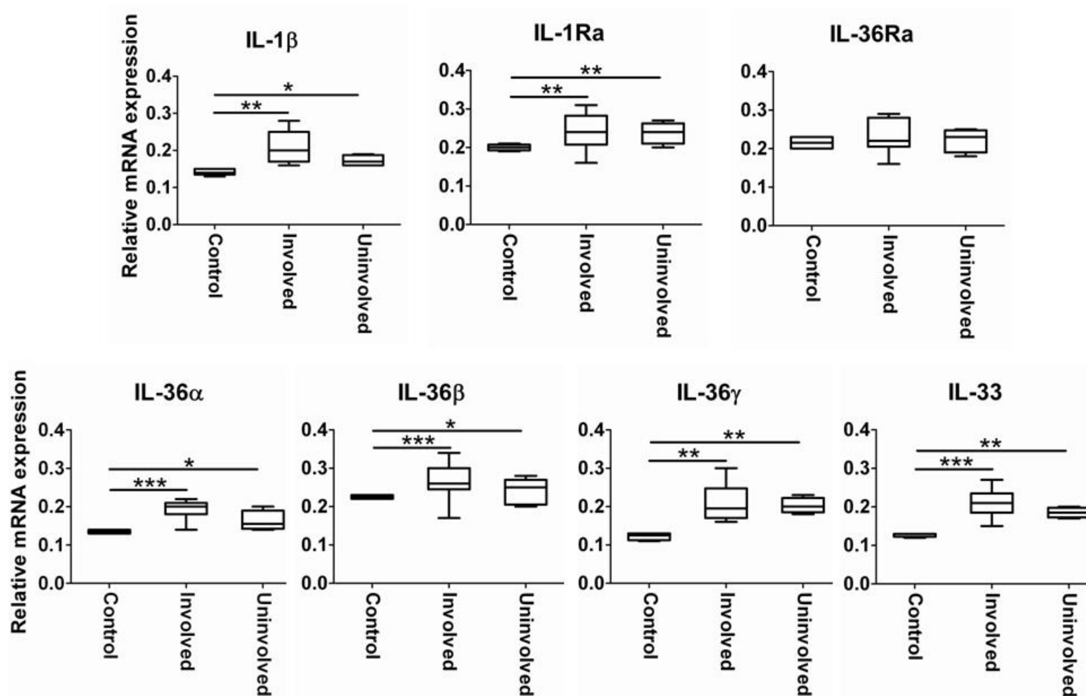

Figure 2. mRNA expression of IL-1 family members in skin biopsies of control skin from normal donors (Control), positive reaction to patch test (Involved) and from uninvolved skin (Uninvolved) of ACD patients. PCR values expressed relative to the housekeeping gene 18S. Data are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively. The line in the box represents the median and whiskers representing min and max. Error bars are not indicated when too small to depict. *p<0.05; **p<0.01; ***p<0.001.

3.2. Immunohistochemical detection of IL-1 family members in ACD

To extend the previous results, we evaluated the immunohistochemical expression of IL-36Ra, IL-36α, IL-36β, IL-36γ and IL-33 in healthy and ACD lesional skin (Fig. 3). IL-36Ra protein levels did not differ between lesional skin and skin from normal donors. Compared with control skin, IL-36α was up-regulated in the spinous layers, IL-36β was up-regulated in suprabasal layers, whereas IL-36γ was up-regulated in all epidermal layers of lesional skin. IL-33 was up-regulated in the basal layers and papillary dermis of ACD eczematous lesions.

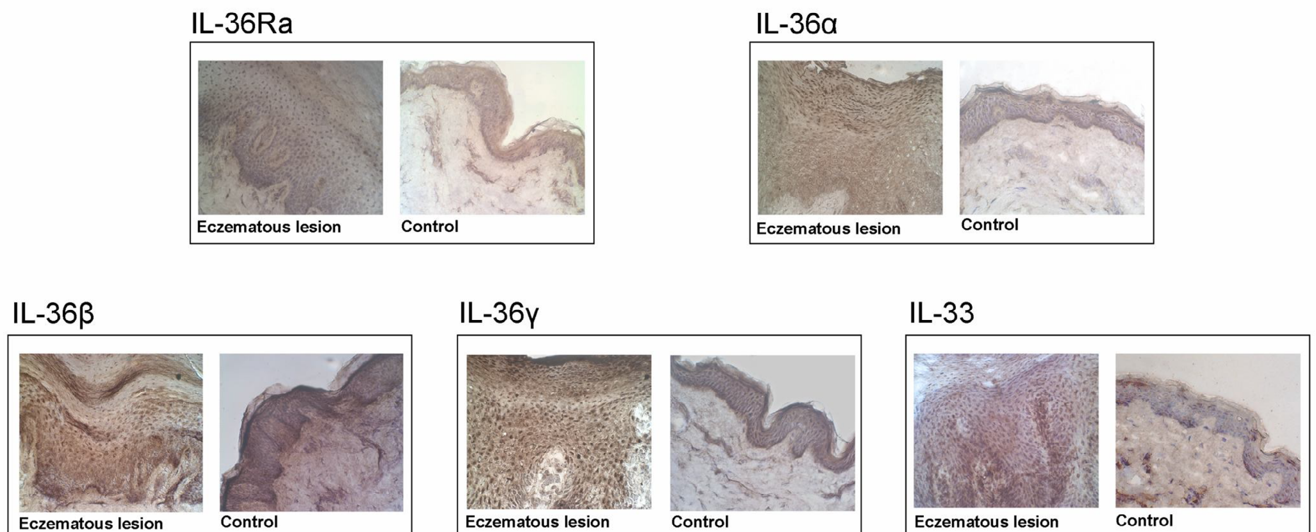

Figure 3. Immunohistochemical detection of IL-36Ra, IL-36α, IL-36β, IL-36γ and IL-33 in eczematous lesion of ACD patients and skin from normal donors (Control). Images X250 magnification.

3.3. *Ex vivo* expression of IL-1 family members in ACD

Given the overexpression of IL-1β, IL-1Ra, IL-36α, IL-36β, IL-36γ and IL-33 mRNA in positive patch test reaction sites, we examined whether these cytokines were increased in an *ex vivo* model of ACD. Uninvolved skin of ACD patients and skin from normal donors was cultured in a transwell system (Trowell, 1954). The skin tissue was placed in a filter with the

epidermis facing upwards at the liquid-air interface. The skin surface was exposed to the specific allergen that had previously elicited the positive patch test reaction or to the vehicle (petrolatum) as a control. Skin tissues obtained from normal donors were exposed to the specific allergen or vehicle. The vehicle did not increase the mRNA expression of any of the interleukins examined (Fig. 4). By contrast, allergen challenge of uninvolved skin of ACD patients significantly increased the mRNA expression of all interleukins examined except IL-36Ra. The effect was specific since allergen challenge did not affect control skin.

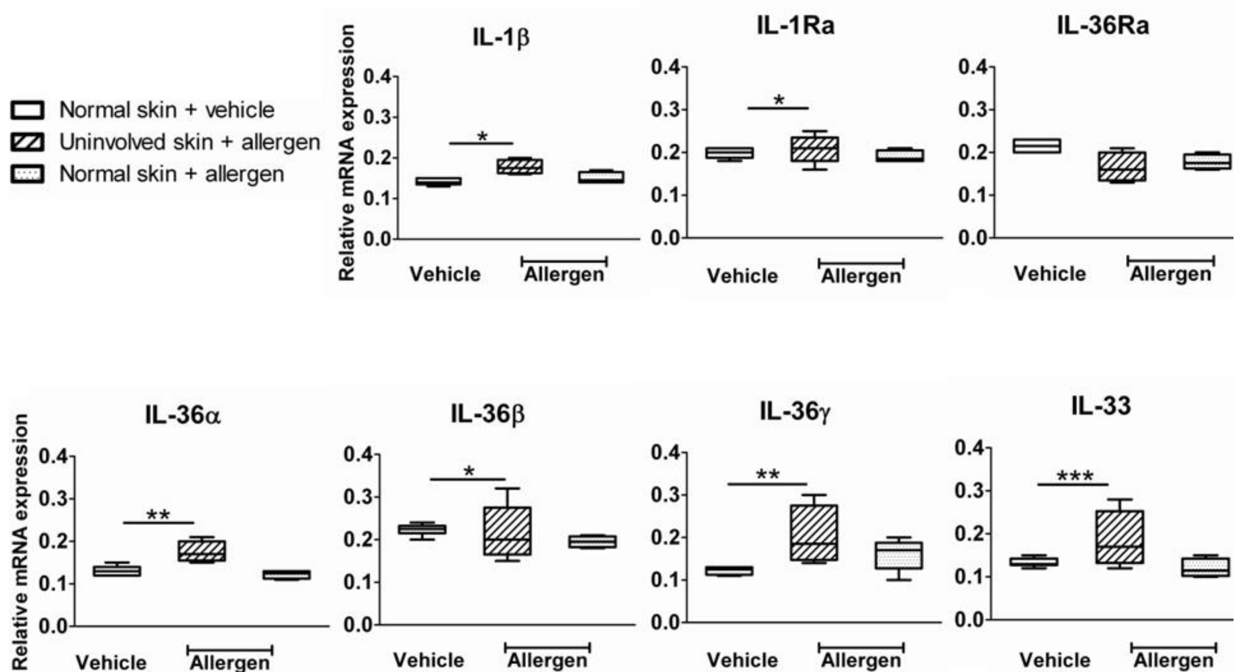

Figure 4. mRNA expression of IL-1 family members in skin from normal donors exposed to the vehicle (Normal skin + vehicle), uninvolved skin of ACD patients exposed to allergen (Uninvolved + allergen) and normal skin exposed to allergen (Normal skin + allergen). PCR values expressed relative to the housekeeping gene 18S. Data are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively; the line in the box represents the median and whiskers representing min and max. Error bars are not indicated when too small to depict.*$p<0.05$; **$p<0.01$; ***$p<0.001$.

3.4. *Ex vivo* exposure to sodium dodecyl sulphate

Skin tissue from normal donors was challenged *in vitro* with increasing non-toxic concentrations of the irritant SDS (2 mM and 4 mM) or with vehicle (H_2O). As shown in figure 5, 2 mM SDS did not affect IL-1β, IL-1Ra, IL-36α, IL-36β, IL-36γ or IL-36Ra expression, whereas exposure to 4 mM SDS enhanced IL-33 expression (p<0.05). The latter result suggests that, in contrast to other members of IL-1 family, IL-33 mRNA can be stimulated by an irritant compound besides by allergens.

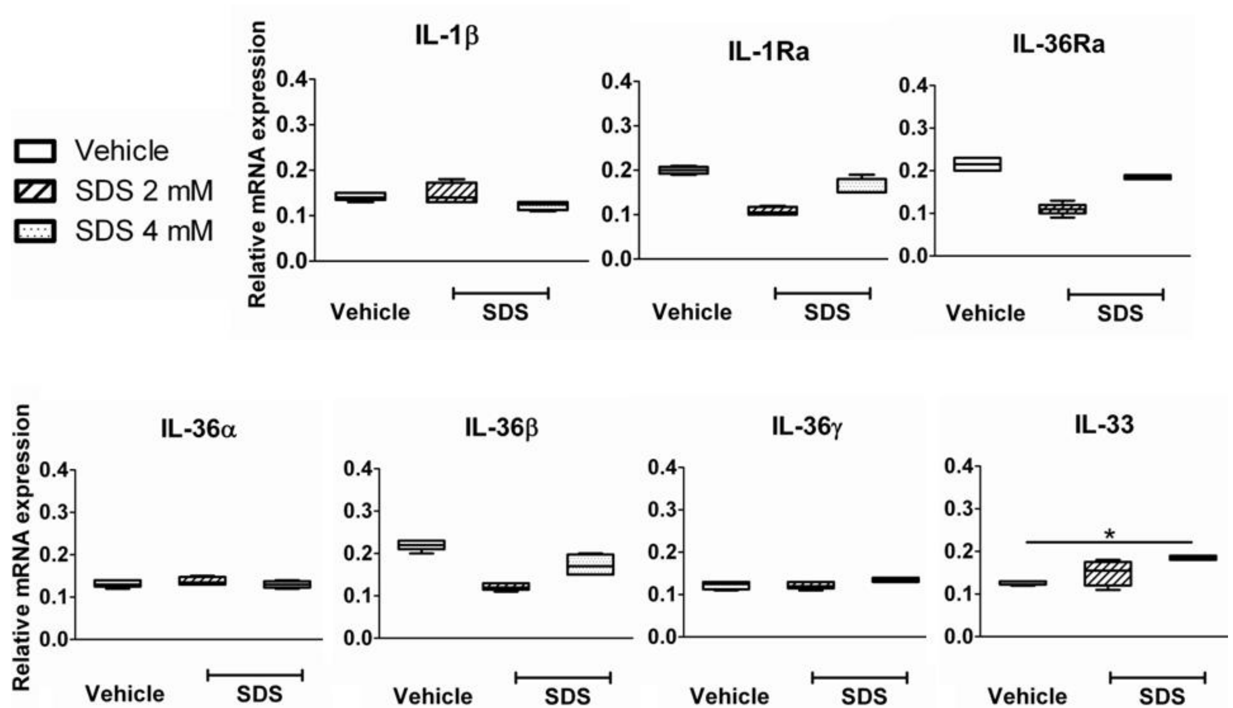

Figure 5. mRNA expression of IL-1 family members in skin from normal donors treated with the vehicle (H_2O) or SDS (2 mM and 4 mM). PCR values expressed relative to the housekeeping gene 18S. Data are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively; the line in the box represents the median and whiskers representing min and max. Error bars are not indicated when too small to depict. *p<0.05.

3.5. *Ex vivo* anti-inflammatory activity of IL-36Ra

Above we report that the mRNA of several members of the IL-1 family (IL-36α, IL-36β, IL-36γ) was constitutively increased in lesional skin of ACD patients, but not the mRNA of their receptor antagonist IL-36Ra (Fig. 2). To evaluate whether lack of this negative regulation could be involved in ACD, we injected 50 µl of rIL-36Ra or the vehicle PBS in the skin biopsy of uninvolved skin of ACD patients and challenged the tissue with the appropriate allergen. We then assessed the mRNA levels of the pro-inflammatory members of the IL-1 family (IL-36α, IL-36β, IL-36γ). We also evaluated IL-8 mRNA, which is known to be induced by the former cytokines (Johnston *et al.*, 2011). Figure 6 shows that injection of rIL-36Ra caused a decrease of IL-36α, IL-36β, IL-36γ and IL-8 mRNA expression compared with PBS-injected skin. These results suggest that rIL-36Ra can modulate the expression of pro-inflammatory members of IL-1 family in this *in vitro* model.

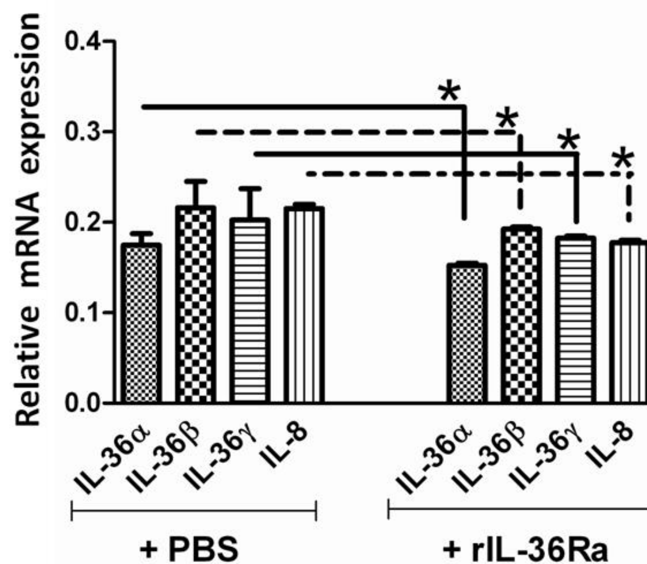

Figure 6. mRNA expression of IL-36α, IL-36β, IL-36γ and IL-8 in uninvolved skin biopsy from ACD patients injected with recombinant rIL-36Ra (50 µl) or PBS and then challenged with the appropriate allergen for 72 h. PCR values expressed relative to the housekeeping gene 18S. Data are displayed as mean ± standard deviation. Error bars are not indicated when too small to depict. *p<0.05.

3.6. IL-1Ra and IL-36Ra expression in peripheral blood mononuclear cells following nickel stimulation

Given the inhibitory effects of IL-36Ra on certain IL-1 cytokines (Sims, Smith, 2010) and the present finding that it was not increased in ACD skin, we investigated the gene expression of two anti-inflammatory members of IL-1 family (IL-1Ra, IL-36Ra) in circulating PBMC. PBMC isolated from ACD patients with a positive patch test to $NiSO_4$ and from healthy donors were exposed *in vitro* to $NiSO_4$. Figure 7 shows that $NiSO_4$ did not modify IL-1Ra or IL-36Ra mRNAs in PBMC of normal donors. By contrast, $NiSO_4$ increased IL-1Ra, but not IL-36Ra mRNA expression, in PBMC of ACD patients.

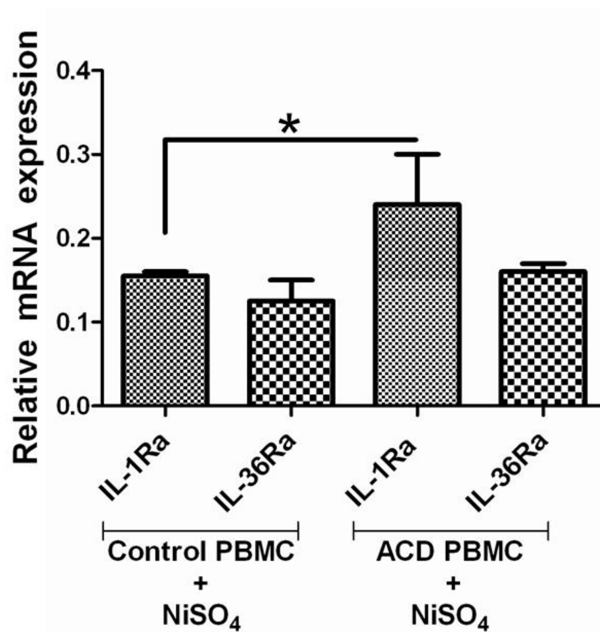

Figure 7. mRNA expression of IL-1Ra and IL-36Ra in PBMC of healthy adults (Control PBMC) and of patients with contact allergy to nickel (ACD PBMC) stimulated with $NiSO_4$. PCR values expressed relative to the housekeeping gene 18S. Data are displayed as mean ± standard deviation. Error bars are not indicated when too small to depict. *$p<0.05$.

3.7. *Ex vivo* IL-33 activity

Above we report that IL-33 mRNA is increased after challenge with allergen (Fig. 4) or 4 mM SDS (Fig. 4). To verify these results, we carried out immunohistochemical staining of skin from healthy donors topically exposed to SDS (4 mM). IL-33 was up-regulated in the basal layers of healthy skin treated with SDS (control + SDS) (Fig. 8a). To determine the relative contribution of IL-33 to skin inflammation in ACD, we investigated whether its neutralization could influence the local inflammatory milieu in ACD. We injected a neutralizing anti-IL-33 antibody (50 µl) or vehicle PBS in uninvolved skin biopsies cultured *ex vivo* before topical exposure to the specific allergen. Figure 8b shows that intradermal injection of PBS does not modify the expression of IL-8 or MCP-1 mRNA. By contrast, anti-IL-33 decreased allergen-induced expression of IL-8 mRNA. No changes were observed regarding MCP-1/CCL2 mRNA.

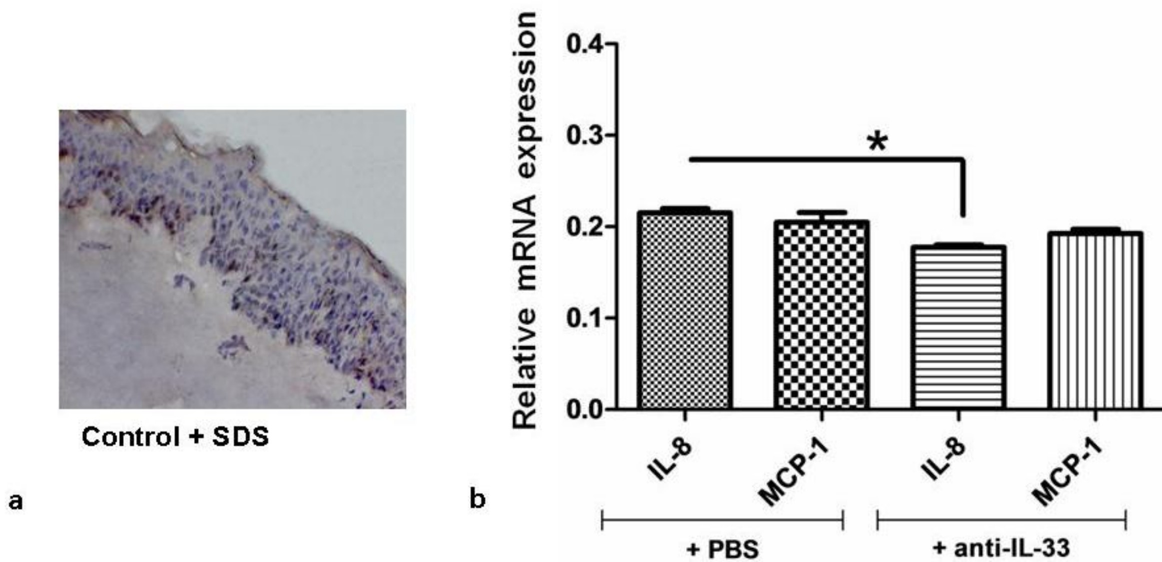

Figure 8. (**a**) Immunohistochemical detection of IL-33 in skin from normal donors treated with SDS 4 mM (Control + SDS). Images X250 magnification. (**b**) Expression of IL-8 and MCP-1 mRNAs in uninvolved skin of ACD patients injected with PBS or anti-IL-33 antibody (50 µl) and then challenged with the appropriate allergen for 72 h. PCR values expressed relative to the housekeeping gene 18S. Data are displayed as mean ± standard deviation. Error bars are not indicated when too small to depict. *p<0.05.

4. DISCUSSION

Skin inflammation in ACD is driven by the coordinated action of a wide spectrum of cytokines, including IL-1 family members (Kaplan *et al.*, 2012). IL-36α (Smith *et al.*, 2000), IL-36β (Smith *et al.*, 2000, Kumar *et al.*, 2000), IL-36γ (Kumar *et al.*, 2000, Busfield *et al.*, 2000) and IL-36Ra (Mulero *et al.*, 1999, Smith *et al.*, 2000), together with their receptor IL-36R, form a novel distinct IL-1 signalling system. Moreover, IL-33 is the most recent addition to the IL-1 family and has potent immune-modulating properties that are mediated by the downstream induction of cytokines and chemokines (Ohno *et al.*, 2011). Given the possible involvement of these new members of the IL-1 family in skin inflammation, we investigated their involvement in human ACD.

We first analyzed skin biopsy specimens obtained from the involved skin of ACD patients, and found that IL-1β, IL-1Ra, IL-36α, IL-36β, IL-36γ and IL-33 mRNA was over-expressed compared to skin from normal donors. These results were extended at protein level by immunohistochemical examination of biopsies of involved skin of ACD patients. The results demonstrated up-regulation of IL-36α, IL-36β and IL-36γ, but not of IL-36Ra, compared to control skin.

Ex vivo skin culture is a widely used technique that enables close monitoring of the events following activation and suppression of the immune system in human skin (Steinstraesser *et al.*, 2009). Using this experimental model we found that, after *ex vivo* challenge, the same IL-1 family members (IL-1β, IL-1Ra, IL-36α, IL-36β, IL-36γ and IL-33) were over-expressed in the uninvolved skin of ACD patients *versus* the skin of normal donors.

The development of ACD crucially depends on providing appropriate ‘danger’ signals to LC (Ouwehand *et al.*, 2008), which are the main IL-1β producers (Enk *et al.*, 1993). Allergen challenge rapidly increases IL-1β mRNA in mouse skin following topical application of the contact hapten (Kermani *et al.*, 2000). This finding supports the hypothesis that IL-1β plays an initial role in skin sensitization. In addition, IL-1β induces keratinocyte production of TNF-α,

which induces LC migration (Cumberbatch *et al*., 1997). Other investigators have argued that in a contact hypersensitivity murine model (IL-1F2-null mouse), IL-1β alone was not essential, which suggests that IL-36γ is involved in contact hypersensitivity (Kumar *et al*., 2000). Here we demonstrate, both *in vivo* and *ex vivo*, that besides IL-1β, also IL-36α, IL-36β and IL-36γ are over-expressed in ACD skin.

IL-33 is constitutively expressed in epidermal keratinocytes and its expression is up-regulated by inflammatory stimuli such as TNF-α or lipopolysaccharides (Schmitz *et al*., 2005). Keratinocytes are positioned in contact with the environment and IL-33 might function as an early warning system at sites of skin inflammation (Dickel et al., 2010). In this study we found constitutive over-expression of IL-33 mRNA in involved skin of ACD patients and in *ex vivo* healthy skin topically exposed to the irritant SDS. These results are consistent with the hypothesis that this cytokine acts as an endogenous warning signal mediating the recruitment of innate immune cells to sites of skin inflammation (Smith, 2010). Our findings extend recent results indicating IL-33 is a danger signal produced in response to tissue damage and UVB (Byrne et al., 2011, Moussion et al., 2008). Interestingly, intradermal injection of anti-IL-33 caused inhibition of IL-8 mRNA, but not of MCP-1/CCL2. IL-8 is involved in ACD as well as in irritancy by exerting chemotactic activity on neutrophils and monocytes (Collington *et al*., 2010, Spiekstra *et al*., 2005). Suppression of IL-8 *ex vivo* by neutralization of IL-33 reinforces the concept that IL-33 acts as an "alarmin".

The IL-1 family includes two inhibitory cytokines: IL-1Ra and IL-36Ra (Sims *et al*., 2010). IL-1Ra competes with IL-1α and IL-1β for binding to IL-1R (Eisenberg *et al*., 1991), whereas IL-36Ra competes with IL-36α, IL-36β and IL-36γ for binding to IL-36R (Towne *et al*., 2004). We found that IL-1Ra is over-expressed in ACD skin, whereas IL-36Ra is not. We obtained similar findings in peripheral blood of nickel-sensitized patients challenged *in vitro* with the allergen. These results highlight a different pattern of expression of two antagonistic IL-1 cytokines in ACD, which suggests that they may modulate cutaneous inflammation differently.

Injection of recombinant human IL-36Ra into involved skin explants of ACD patients caused inhibition of IL-36α, IL-36β, IL-36γ and IL-8 mRNAs. These results might indicate that a lack of over-expression of IL-36Ra could be, at least in part, responsible for the over-expression of IL-36α, IL-36β, IL-36γ in ACD patients. Over-expression of IL-36α, IL-36β and IL-36γ in the skin of ACD patients can induce Th-17 cytokines that participate in the physiopathology of ACD (Zhao *et al.*, 2009). The role of IL-36s and IL-36Ra in ACD is also supported by experiments conducted with transgenic mice deficient in IL-36Ra (Blumberg *et al.*, 2007).

In conclusion, our results highlight the complexity of the involvement of pro-inflammatory and antagonistic members of the IL-1 family in the pathogenesis of ACD. In particular we found an over-expression at both molecular and protein level of IL-1β and IL-36α, IL-36β, IL-36γ in the skin of ACD patients where these cytokines can play a pro-inflammatory role. We also detected over-expression of IL-33 in both allergic and irritant conditions suggesting that this cytokine might function as an early warning system at the site of skin damage. While IL-1Ra was increased in ACD skin, IL-36Ra was unchanged, which suggests that the activity of IL-36α, IL-36β and IL-36γ is "unopposed" and therefore that these cytokines can drive skin inflammation. This observation prompts the speculation that IL-36Ra might be a candidate for ACD therapy.

This study has started to shed light on the complexity of the possible interactions among different agonistic and antagonistic members of the IL-1 family in ACD. The characterization of the roles of these cytokines in skin inflammation in ACD requires additional experiments.

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