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Title: The balance between pro- and anti-inflammatory cytokines is crucial in human allergic contact dermatitis pathogenesis: the role of IL-1 family members.

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Abstract

The interleukin (IL)-1 family includes 11 members that are important in inflammatory processes. It includes various agonists and two antagonists, IL-1Ra and IL-36Ra. **Our aim was to investigate** whether the IL-1 family is involved in allergic contact dermatitis (ACD). The expression of IL-1 family members was evaluated by PCR and immunohistochemistry in the positive patch test reaction site (involved skin), and in the uninvolved skin of ACD patients. We also examined these cytokines in an *ex vivo* model of ACD. The antagonistic activity of IL-36Ra was evaluated by injecting recombinant IL-36Ra in uninvolved skin biopsies of ACD patients. IL-1Ra and IL-36Ra expression was quantified in mononuclear cells of nickel-sensitized patients challenged *in vitro* with nickel. IL-33-involvement in ACD was investigated by intradermal injection of anti-IL-33 in the uninvolved skin of patients *ex vivo*. Results showed that IL-1 β , IL-1Ra, IL-36 α , IL-36 β , IL-36 γ and IL-33 expression, but not IL-36Ra expression was enhanced in ACD-involved skin. Immunohistochemical analysis and *ex vivo* skin cultures confirmed these results. Injection of anti-IL-33 in ACD-uninvolved skin inhibited IL-8 expression, whereas IL-36Ra inhibited IL-36 α , IL-36 β , IL-36 γ and IL-8 expression. Nickel induced IL-1Ra expression in lymphocytes of nickel-sensitized patients. **Hence**, various IL-1 agonists and antagonists may be involved in ACD pathogenesis.

Key words: allergic contact dermatitis; IL-1 family; IL-1; IL-33; IL-36

Introduction

Allergic contact dermatitis (ACD) is a common skin disease caused by delayed-type hypersensitivity responses to low molecular weight contact allergens (haptens) that come into contact with the skin (1, 2). Sensitization to contact allergens results from their uptake by antigen-presenting cells in the skin such as Langerhans cells (LC) or dermal dendritic cells (DC) followed by priming of allergen-specific T cells in the draining lymph nodes (3). Upon

interactions with skin-associated lymphoid tissue, these hapten-bearing antigen-presenting cells 'educate' naïve T lymphocytes to become allergen-specific memory and effector T cells (4). During the elicitation phase, allergen-specific T lymphocytes are reactivated in the local lymph nodes and migrate to the challenge site to produce cytokines, chemokines and cytotoxic damage to the skin, resulting in the clinical signs and symptoms of ACD (1, 5).

The interleukin (IL)-1 family is a growing group of cytokines, consisting at present of at least 11 members, that plays an important role in immune regulation and inflammatory processes (6). It is closely linked to the innate immune response and is primarily associated with acute and chronic inflammation. IL-1 α (alternative name IL-1F1) and IL-1 β (IL-1F2) both bind to IL-1R1, whereas IL-1F3 (IL-1Ra) has an antagonistic effect on this receptor. IL-18 (IL-1F4) exerts pro-inflammatory activity by binding to IL-18R α . Cytokines IL-36 α , IL-36 β and IL-36 γ (alternative names IL-1F6, IL-1F8 and IL-1F9, respectively) bind to the IL-36 receptor (IL-36R, also called IL-1Rp2 or IL-1RL2) and initiate a pro-inflammatory signal (7). An IL-36 receptor antagonist (IL-36Ra), also termed IL-1F5, inhibits signalling by binding to IL-36R (7). IL-33 (IL-1F11) binds to ST2 receptor thereby triggering pro-inflammatory activity (8). The functions of IL-1F7 and IL-1F10 are not yet well defined (7). IL-1 β and IL-18 are produced by LC after activation by haptens (9) and they up-regulate the expression of co-stimulatory molecules by LC thereby enabling their migration out of the skin to the draining lymph node (10). IL-1 α and IL-33 are known to be involved in contact hypersensitivity (11, 12).

Several cytokines, including members of the IL-1 family, play a fundamental role in the sensitization, elicitation and resolution phases of experimental contact hypersensitivity reactions (13-16). IL-1 is involved in the early inflammatory events that dictate the

development and severity of ACD (17). Genetic susceptibility accounts for differences in host sensitivity to allergens, and antagonism to the pro-inflammatory cytokine IL-1 tends to be attenuated in patients who carry an IL-1Ra gene polymorphism (18). In addition, IL-18 has been shown to be a mediator of ACD in both human and murine models (19-21).

It remains to be established whether the new IL-1 family members are expressed in the skin of patients with ACD. To better define the role of this family in skin inflammation, we assessed the expression of IL-1 β , IL-1Ra, IL-36s and IL-33 by real-time PCR and immunohistochemistry in biopsies of normal donors, **in the uninvolved as well as involved skin of patients affected by ACD.**

Materials and methods

The study population consisted of 25 subjects (mean age: 45.3 ± 2.7 years) with at least one positive reaction to patch test (Table S1). 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 (22). Results were recorded as negative, doubtful, weakly positive (1+), moderate reaction (2+) and strong reaction (3+).

In addition, skin biopsies were collected from 10 healthy subjects and used as controls.

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.

In vivo expression of IL-1 family members in ACD

Skin biopsies (3 mm each) from involved and uninvolved sites (clinically normal skin) were collected from subjects with at least one positive reaction to patch test, and used for *in*

vivo mRNA examination through real-time PCR. **In order to avoid inflammatory infiltrate contamination, uninvolved biopsy samples were obtained from clinically normal skin always with a distance of at least 10 cm from the positive reaction.** Skin specimens from normal donors (control) who had undergone plastic surgery and without a history of contact sensitization served as controls.

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 IL-33 (0.5 $\mu\text{g/ml}$, clone Nussy-1, Alexis Biochemicals, San Diego, CA), IL-36Ra (5 $\mu\text{g/ml}$ R&D System Inc., Minneapolis, MN), IL-36 β (5 $\mu\text{g/ml}$ R&D System Inc, Minneapolis, MN), IL-36 α (15 $\mu\text{g/ml}$ R&D System Inc, Minneapolis, MN), IL-36 γ (15 $\mu\text{g/ml}$ R&D System Inc, Minneapolis, MN) and incubated overnight at 4°C .** In parallel, skin specimens were incubated with specific isotype control antibodies (Mouse IgG1 Isotype Control, Mouse IgG2B Isotype Control, Goat IgG Control, R&D System Inc, Minneapolis, MN used at the same concentration as the corresponding primary antibody. 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. For immunohistochemistry staining intensity, histology images were analyzed using the

TMARKER (<http://comp-path.inf.ethz.ch>) image analysis software after color deconvolution according to the standardized protocol (23). **Mean intensity values of all analyzed interleukins were calculated for each patient and control.**

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 μ m; 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 μ g/ml streptomycin, GIBCO) (24). 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% CO₂. 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 also 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.

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 the vehicle (H₂O). The irritation assay was performed as follows: IQ chamber filter paper discs

(Chemotechnique diagnostics, Velling, Sweden), impregnated with 25 µl of SDS non-cytotoxic concentrations (2 mM and 4 mM) (Sigma Chemical Co., St. Louis, MO) or vehicle only (H₂O), were applied topically to the upwards surface of cultured skin for 72 h at 37° C (25).

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. **50 µl of recombinant IL-36Ra (rIL-36Ra; 1.25 µg/mL, R&D System, Inc, Minneapolis, MN) or the vehicle only (PBS) were injected into the uninvolved specimens.**

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). Cells 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 2×10^6 in 12-well plates. Cells were stimulated with NiSO₄ (10^{-5} M) and incubated at 5% CO₂ at 37° C.

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

Skin specimens obtained from 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.

Ex vivo activity of IL-33

Skin biopsies from uninvolved skin of 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. **50 μ l of anti-IL-33 polyclonal antibody (0.2 μ g/mL, Abcam, Cambridge, UK) or the vehicle only (PBS) were injected into the uninvolved specimens.**

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

RNA was extracted from every skin biopsy specimen (RNeasy Mini Protocol, Qiagen Valencia, CA) 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-1 β , IL-1Ra, IL-36Ra, IL-36 α , IL-36 β , IL-36 γ , IL-33, IL-8 and monocyte chemoattractant protein-1 (MCP-1/CCL2). Each PCR was performed 2 times, each time in triplicate. PCR protocol and product quantification for 18S ribosomal RNA were performed as reported previously (26). 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. The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of 18S reference gene in the same sample. Fold induction of gene expression was calculated using the $\Delta\Delta$ CT method as described previously (27). Results obtained from each PCR were pooled and statistically analyzed.

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.

Results

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

The recent identification of new members of the IL-1 family prompted us to investigate 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).

The expression of IL-1 β ($p < 0.01$) IL-1Ra ($p < 0.05$) and IL-33 ($p < 0.01$) was significantly higher in involved and uninvolved skin of ACD patients than in skin from normal donors (Fig. 1). The expression of IL-36 α ($p < 0.01$), IL-36 β ($p < 0.01$) and IL-36 γ ($p < 0.05$) was significantly higher in involved skin of ACD patients than in skin from normal donors. By contrast, the expression of the anti-inflammatory IL-36Ra cytokine in involved and uninvolved skin did not differ from that in control skin (Fig. 2).

Immunohistochemical detection of IL-1 family members in ACD

We also evaluated the immunohistochemical expression of IL-36Ra, IL-36 α , IL-36 β , IL-36 γ 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 (Fig. 3a). Compared with control skin, IL-36 α was up-regulated in the spinous layers (Fig. 3b), IL-36 β was up-regulated in suprabasal layers (Fig. 3c), whereas IL-36 γ was up-regulated in all epidermal layers of lesional skin (Fig. 3d). IL-33 was up-regulated in the basal layers and papillary dermis of

ACD eczematous lesions (Fig. 3e). **Immunohistochemical staining intensities of all analyzed interleukins confirmed the results above reported,** showing statistically significant labelling for IL-36 α , IL-36 β , IL-36 γ and IL-33 with the only exception of IL-36Ra. Results of staining estimation are given in Table S2.

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

Given the overexpression of IL-1 β , IL-1Ra, IL-36 α , IL-36 β , IL-36 γ 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 **were** cultured in a transwell system (22). 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. S1). By contrast, allergen challenge of uninvolved skin of ACD patients significantly increased the mRNA expression of all interleukins examined except IL-36Ra. **The lack of effects of the allergen challenge on control skin from normal donors may exclude a contact allergen direct triggering of immune pathways in favour of the activation of an allergic one.**

***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 (H₂O). As shown in figure S2, 2 mM SDS did not affect IL-1 β , IL-1Ra, IL-36 α , IL-36 β , IL-36 γ 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 the IL-1 family, IL-33 mRNA can be stimulated by an irritant compound besides by allergens, at least at 72h.

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

Above we reported that the mRNA of several members of **IL-1 family** (IL-36 α , IL-36 β , IL-36 γ) was increased in lesional skin of ACD patients, **whereas no increase was found for their receptor antagonist IL-36Ra** (Fig. 1 and Fig. 2). To evaluate whether **the** lack of this negative regulation could be involved in ACD, we injected 50 μ l 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-36 α , IL-36 β , IL-36 γ). We also evaluated IL-8 mRNA, which is known to be induced by the above indicated cytokines (28). Figure 4a shows that injection of rIL-36Ra caused a decrease of IL-36 α , IL-36 β , IL-36 γ 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.

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 (7) 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 NiSO₄ and from healthy donors were exposed *in vitro* to NiSO₄. Figure S3 shows that NiSO₄ did not modify the expression of IL-1Ra or IL-36Ra mRNAs in PBMC of normal donors. By contrast, NiSO₄ increased IL-1Ra, but not IL-36Ra mRNA expression, in PBMC of ACD patients. These findings, although

limited by multiple cell contamination, provide a clue concerning the involvement of circulating cells in the regulation of IL-1 family members in ACD.

***Ex vivo* IL-33 activity**

Above we reported that IL-33 mRNA **was** increased after challenge **either with allergen (Fig. S1) or with 4 mM SDS** (Fig. S2). 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. 4b**). 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 μ l) or PBS in uninvolved skin biopsies cultured *ex vivo* before topical exposure to the specific allergen. Figure 4c shows that intradermal injection of PBS did not modify the expression of IL-8 or MCP-1/CCL2 mRNA. By contrast, anti-IL-33 decreased allergen-induced expression of IL-8 mRNA. No changes were observed regarding MCP-1/CCL2 mRNA.

Discussion

Skin inflammation in ACD is driven by the coordinated action of a wide spectrum of cytokines, including IL-1 family members (1). IL-36 α (29), IL-36 β (29, 30), IL-36 γ (30, 31) and IL-36Ra (29, 32), 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 (8). 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-1 β , IL-1Ra, IL-36 α , IL-36 β , IL-36 γ 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-36 α , IL-36 β and IL-36 γ , but not of IL-36Ra, compared to control skin. We have previously reported that IL-1 family members were increased in another pro-inflammatory condition, such as psoriasis (33, 34). **In particular, a striking increase was observed in the expression of pro-inflammatory members respect to the anti-inflammatory ones (33).**

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 (35). Using this experimental model we found that, after *ex vivo* challenge, the same IL-1 family members (IL-1 β , IL-1Ra, IL-36 α , IL-36 β , IL-36 γ 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 (36), which are the main IL-1 β producers (37). **In addition, IL-1 β induces LC migration by promoting the production of TNF- α by keratinocyte (38). However, studies carried out in a contact hypersensitivity murine model (IL-1F2-null mouse) indicate that IL-1 β alone is not essential, and suggest that IL-36 γ is involved in contact hypersensitivity (30).** Allergen challenge rapidly increases IL-1 β mRNA in mouse skin following topical application of the contact hapten (38), and this finding supports the hypothesis that IL-1 β plays an initial role in skin sensitization. Indeed, IL-1 β , as well as IL-18, can be produced by KC other than LC, thus providing a link between innate and adaptive immunity in ACD (40,

41). These interleukins can be cleaved by caspase-1 and secreted by KC through an inflammasome-mediated manner during the early phase of ACD. In addition, the increase of IL-1 β is also sustained by toll like receptor (TLR) signalling, implicating that the TLR and inflammasome systems can work coordinately to induce the release of pro-inflammatory cytokines (1) TLR senses microbial pathogens and endogenous ligands, but recent studies have shown that contact allergens can activate these receptors in an indirect manner by induction of endogenous activators (42). However, recent studies have shown that inorganic molecules such as Ni²⁺ ions can directly activate the TLR-inflammasome pathway, which is translated in a fast activation of innate immune response (43). **Consistently with these observations, here we demonstrated, both *in vivo* and *ex vivo*, that IL-1 β was over-expressed in ACD skin**, and this could be explained by the rapid hapten-induced activation of the innate immune pathways. **Of note, we also observed that allergen challenge on control skin from normal donors did not induce IL-1 β enhancement**, favouring the hypothesis that activation of the allergic pathway might underlie, at least in part, IL-1 β regulation.

Besides IL-1 β , we found that also IL-36 α , IL-36 β , IL-36 γ and IL-33 were over-expressed in ACD skin. If on the one hand the TLR-inflammasome activation can partially explain the regulation and the rapid release of these IL-1 family members, on the other hand little is known about the relationship between the newly discovered IL-1 family members and the inflammasome. A recent study suggests that truncation of IL-36 α , IL-36 β and IL-36 γ dramatically enhances their activity, but the proteases responsible for their post-translational regulation have not yet been identified (44). Regarding IL-33, its mRNA is translated without a signal sequence for secretion and cannot be released by the processing and secretion mechanism shared by IL-1 β and IL-18 as IL-33 is not a substrate of caspase-1 and does not

require proteolysis for activation (45). Indeed, IL-33 is produced as a propeptide, is cleaved by caspase 3, 7 and calpain, although the uncleaved protein is now supposed to be the active form (46, 47)

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 (48). **In fact,** IL-33 is constitutively expressed in epidermal keratinocytes and its expression is up-regulated by inflammatory stimuli such as TNF- α or lipopolysaccharides (45). Keratinocytes are positioned in contact with the environment and IL-33 might function as an early warning system at sites of skin inflammation (49). IL-33 is not the unique IL-1 family member acting as an endogenous danger signal. **This role has been also described for IL-1 α ;** this interleukin is biologically active as precursor, and it is mainly released after tissue injury or necrosis (6). Consistent with our results regarding IL-33, Ulfgren *et al* observed IL-1 α -positive cells in skin exposed to both irritant or allergic compounds (50). 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 (51, 52). **Suppression of IL-8 *ex vivo* by neutralization of IL-33 reinforces the concept that IL-33 acts as an “alarmin” produced in response to tissue damage** (53, 54).

The IL-1 family includes two inhibitory cytokines: IL-1Ra and IL-36Ra (7). IL-1Ra competes with IL-1 α and IL-1 β for binding to IL-1R (55), whereas IL-36Ra competes with IL-36 α , IL-36 β and IL-36 γ for binding to IL-36R (56). 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 uninvolved skin explants of ACD patients caused inhibition of IL-36 α , IL-36 β , IL-36 γ 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 induction of IL-36 α , IL-36 β , IL-36 γ in ACD patients. Moreover, over-expression** of IL-36 α , IL-36 β and IL-36 γ in the skin of ACD patients can induce Th-17 cytokines that participate in the physiopathology of ACD (57). **In addition**, the role of IL-36s and IL-36Ra in ACD is also supported by experiments conducted with transgenic mice deficient in IL-36Ra (58).

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-1 β and IL-36 α , IL-36 β , IL-36 γ 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-36 α , IL-36 β and IL-36 γ is “unopposed” and therefore that these cytokines can drive skin inflammation. As the study of new therapeutic agents is becoming more and more important for the treatment of inflammatory dermatoses as ACD (59-61), 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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Figure legends

Fig. 1. mRNA fold expression of IL-1 β , IL-1Ra and IL-33 in skin biopsies of control skin from normal donors (Control), positive reaction to patch test (Involved) and uninvolved skin (Uninvolved) of ACD patients. PCR values are expressed as relative fold expression normalized to the housekeeping gene 18S respect to control skin from normal donors. Data are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively and representative of 2 PCR each performed in triplicate. The line in the box represents the median and whiskers representing min and max. Error bars are not indicated when too small to depict. * $p < 0.05$; ** $p < 0.01$.

Fig. 2. mRNA fold expression of IL-36 α , IL-36 β , IL-36 γ and IL-36Ra in skin biopsies of control skin from normal donors (Control), positive reaction to patch test (Involved) and uninvolved skin (Uninvolved) of ACD patients. PCR values are expressed as relative fold expression normalized to the housekeeping gene 18S respect to control skin from normal donors. Data are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively and representative of 2 PCR each performed in triplicate. The line in the box represents the median and whiskers representing min and max. Error bars are not indicated when too small to depict. * $p < 0.05$; ** $p < 0.01$.

Fig. 3. Immunohistochemical detection of IL-36Ra (a), IL-36 α (b), IL-36 β (c), IL-36 γ (d) and IL-33 (e) in eczematous lesion of ACD patients (X250 magnification) and skin from normal donors (Control, X400 magnification). Each picture is representative of data from 15 different patients affected by ACD and 10 normal donors.

Fig. 4. (a) mRNA fold expression of IL-36 α , IL-36 β , IL-36 γ and IL-8 in uninvolved skin biopsy from ACD patients injected with recombinant rIL-36Ra (50 μ l) or PBS and then challenged with the appropriate allergen for 72 h. (b) Immunohistochemical detection of IL-33 in skin from normal donors treated with SDS 4 mM (Control + SDS). Images X250 magnification. The picture is representative of data from 5 independent experiments. (c) Expression of IL-8 and MCP-1 mRNAs in uninvolved skin of ACD patients injected with PBS or anti-IL-33 antibody (50 μ l) and then challenged with the appropriate allergen for 72 h. PCR values are expressed as relative fold expression normalized to the housekeeping gene 18S respect to unstimulated control skin from normal donors. Data are displayed as mean $\pm$ standard deviation, representative of 2 PCR each performed in triplicate. Error bars are not indicated when too small to depict. * $p < 0.05$.

Fig. S1. mRNA fold 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 are expressed as relative fold expression normalized to the housekeeping gene 18S respect to unstimulated control skin from normal donors. Data are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively and representative of 2 PCR each performed in triplicate; the line in the box represents the median and whiskers representing min and max. Error bars are not indicated when too small to depict. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. S2. mRNA fold expression of IL-1 family members in skin from normal donors treated with the vehicle (H₂O) or SDS (2 mM and 4 mM). PCR values are expressed as relative fold expression normalized to the housekeeping gene 18S respect to unstimulated control skin from normal donors. Data are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively and representative of 5 independent experiments, each performed in duplicate; the line in the box represents the median and whiskers representing min and max. Error bars are not indicated when too small to depict. * $p < 0.05$.

Fig. S3. mRNA fold 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 NiSO₄. PCR values are expressed as relative fold expression normalized to the housekeeping gene 18S respect to unstimulated PBMC from normal donors and ACD subjects. Data are displayed as mean $\pm$ standard deviation, representative of 5 independent experiments, each performed in duplicate. Error bars are not indicated when too small to depict. * $p < 0.05$.







