DOTTORATO DI RICERCA IN
MEDICINA CLINICA E SPERIMENTALE
CURRICULUM IN SCIENZE IMMUNOLOGICHE E DERMATOLOGICHE

XXIX Ciclo
Coordinatore: Prof. Gianni Marone

TESI DI DOTTORATO

TITOLO

The role of interleukin-36 in inflammatory skin diseases

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References
Interleukin (IL)-36 cytokines are new members of the IL-1 family, that include pro-inflammatory factors, IL-36α, IL-36β and IL-36γ, and a natural receptor antagonist IL-36Ra. IL-36 cytokines are expressed in a specific manner by monocytes/macrophages, dendritic cells (DCs), T cells subsets, keratinocytes, Langerhans cells, and mucosal epithelium. Since IL-36 cytokines are predominantly expressed in keratinocytes it is not surprising that specifically skin disorders have been explored for associations with these cytokines. Several reports have found that IL-36α and IL-36γ are up-regulated in psoriatic and allergic contact dermatitis skin. Over recent years much has been learned on their important functions in the regulation of immune response and, especially, on their involvement in many inflammatory conditions. In this study, we wanted to elucidate the role of these mediators in the pathogenesis of most common inflammatory skin diseases such as, psoriasis, allergic contact dermatitis and polymorphic light eruption.
I CHAPTER

IL-36 cytokines

The interleukins (IL)-36 are members of the IL-1 superfamily, including 3 agonistic cytokines, namely IL-36α, IL-36β and IL-36γ, previously known as IL-1F6, IL-1F8, and IL-1F9, and a natural inhibitor, IL-36Ra, previously termed IL-1F5. IL-36 were originally identified by a number of groups simultaneously through analysis of the human genome in search of gene sequences predicted to encode homologs of IL-1.1–5 IL-36α, β, γ elicit their proinflammatory effects through binding IL-36R, which subsequently forms a heterodimer with the IL-1R accessory protein (IL-1RAcP) to activate intracellular signaling pathways. The IL-1RAcP is also shared by the related IL-1 family receptors for IL-33 and IL-1α/β, with the IL-36R chain alone responsible for ligand specificity. Similar to these related receptors, the IL-36R chain is composed of an extracellular ligand-binding domain, a transmembrane helix, and an intracellular Toll/IL-1 receptor (TIR) domain required for the initiation of intracellular signaling cascades.6 The extracellular portion consists of three Ig-like domains, and the specific residues required for ligand binding have recently been identified.7 Upon activation, the IL-36R initiates distinct signaling pathways through the recruitment of the MyD88 adaptor protein to TIR domains present on both the IL-36R and IL-1RAcP. As with other IL-1 family members, this leads to the subsequent activation of the MAPK pathways mediated by JNK and ERK1/2, as well as NF-KB–dependent transcription, which combine to promote proinflammatory gene expression.8 Similarly, IL-36Ra binds to IL-36R, but it prevents binding of IL-36α, IL-36β, and IL-36γ. Recent structural analysis of how IL-36Ra interacts with the receptor complex indicate that it acts as a classical antagonist through competitive binding to the extracellular Ig domains of the IL-36R chain, thereby preventing ligand binding and subsequent recruitment of the IL-1RAcP.7,9 As a result, no downstream signaling is initiated through the IL-36R. Furthermore,
it has also been demonstrated that another IL-1 family member, IL-38, can also directly bind and act as an antagonist of the IL-36R.\textsuperscript{10}

The IL-36 cytokines are clustered on human chromosome 2 in the same locus that includes the genes for all other IL-1 family cytokines except for IL-18 and IL-33. The IL-36R also lies in the IL-1R family locus on human chromosome 2 and is flanked by IL-1R1 and IL-1R2 on the centromeric side and IL-33R, IL-18R, and IL-18RAP (accessory protein) on the telomeric side.\textsuperscript{11-13} IL-36 cytokines, such as all IL-1 family members except for IL-1Ra, are synthesized without a signal peptide and therefore, are not secreted via the endoplasmic reticulum-Golgi pathway. It is unclear how IL-36 cytokines are released from cells; however, it is clear that post-translational processing is required for full agonist or antagonist activity.
of all IL-36 members.\textsuperscript{14} Truncation of IL-36 members must occur at precisely the right location to generate the active form, whereas removal of 1 more or less amino acids results in a protein with activity comparable with the full-length protein. The enzyme or enzymes required for processing of IL-36 cytokines are unknown, and there are no aspartate residues or other features suggesting a cleavage site for caspase-1.\textsuperscript{12,13} IL-36 family cytokines are expressed in several tissues and by various immune and parenchymal cell subsets. Individual IL-36 cytokines are expressed in a specific manner by monocytes/macrophages, dendritic cells (DCs), T cells subsets, keratinocytes, Langerhans cells, and mucosal epithelium.\textsuperscript{15-17} They are strongly up-regulated in the skin by many agents, including cytokines, TLR agonists, or by pathologic conditions. Indeed, IL-36α and γ are highly expressed in inflamed skin from psoriasis patients,\textsuperscript{18-20} as well as lesional skin from patients with atopic dermatitis. Furthermore, expression of IL-36α, β, and γ has been described in colonic epithelial cells from patients with inflammatory bowel disease (IBD),\textsuperscript{21,22} whereas IL-36γ expression is strongly induced in bronchial epithelium exposed to inflammatory stimuli.\textsuperscript{23}

**IL-36 and their immune function**

IL-36 cytokines act as a crucial link in translating innate immune responses into the appropriate adaptive immune response. In particular, IL-36 exerts strong stimulatory effects on dendritic cells (DCs) and T-cells that play a critical role in the interface between innate and adaptive immune responses.\textsuperscript{24} IL-36 agonists are expressed by murine DC subsets in response to inflammatory stimuli; in addition they promote the maturation of DCs, up-regulating markers of activation such as CD80, CD86 and MHCII.\textsuperscript{16} Murine as well as human DC subsets have been shown to express IL-36R and respond to stimulation with IL-36 ligands.\textsuperscript{16,25} In contrast to IL-1, IL-36 can provide a relatively potent proinflammatory stimulus to murine bone marrow–derived DCs, leading to the induced expression of chemokines, such as CXCL1, CCL1, CXCL10, and CCL3, as well as proinflammatory
cytokines, including TNFα, IL-6, and granulocyte-macrophage colony-stimulating factor. IL-36α and IL-36β are constitutively expressed by human and murine T cells, respectively. Regarding IL-36R expression, there is an important difference between human and mouse cells. Indeed, mouse T cells express IL-36R and respond directly and indirectly to IL-36 stimulation. IL-36α, IL-36β, and IL-36γ have a direct effect on mouse CD4+ T cells and splenocytes, inducing proinflammatory cytokines. In addition, IL-36 has a role in the induction of T cell polarization. IL-36β, like IL-18, acts in combination with IL-12 to induce the in vitro differentiation of Th0 cells into IFNγ-producing Th1 cells. The influence of IL-36 on Th1 differentiation is completely dependent on IL-2 induction. In contrast, the effect of IL-36 on Th0 cell proliferation and survival is largely independent of IL-2. Vigne et al. also demonstrated that CD4+ T cells constitutively express IL-36β, which can act in an autocrine fashion to promote cell survival as well as Th1 differentiation. Although IL-36 cytokines can potently induce Th1 responses, they do not appear to play a significant role in the host response to mycotic infection. Human peripheral blood mononuclear cells that are stimulated with C. albicans, a potent inducer of the Th17 response in humans, produce less IL-17 and IL-22 in the presence of IL-36Ra. IL-36 cytokines can modulate the immune system primarily through their effects on antigen-presenting cells, such as DCs and in this way can polarize T helper responses.
II CHAPTER

IL-36 and diseases

Unsurprisingly, in tandem with the emergence of data identifying the proinflammatory mechanisms through which IL-36 family members exert their effects, evidence of their central role as regulators of inflammatory disease has begun to accumulate.\textsuperscript{29} Indeed, IL-36 might also play a significant role in joint disease. Remarkably, IL-36β is expressed in mouse and human joints\textsuperscript{30} and IL-36α was found to be up regulated in joints of patients with psoriatic and rheumatoid arthritis. Anyhow, no differences were detected for IL-36β at serum level between healthy human volunteers, and subjects affected by RA. A potential role for the IL-36 family as mediators of intestinal inflammation in the context of inflammatory bowel disease (IBD) has also recently emerged.\textsuperscript{31} In contrast to the skin, where IL-36 appears to act exclusively in a proinflammatory capacity, the role of these cytokines in the gut appears more complex. Preliminary data provide evidence that IL-36 cytokines might play a role in obesity. Indeed, IL-36Ra is also expressed in differentiated pre-adipocytes, and can be downregulated by TNF-α, a proinflammatory cytokine.\textsuperscript{32} Moreover, IL-36α and IL-36β can induce the expression of pro-inflammatory genes in mature adipocytes.\textsuperscript{33} Accumulating data also indicate that IL-36 family members may play a prominent role also in inflammatory diseases in the lung.\textsuperscript{34}

IL-36 and skin diseases

Since IL-36 cytokines are predominantly expressed in epithelial cells and in particularly in keratinocytes it is not surprising that different skin disorders have been explored for associations with these cytokines. Several reports have found that IL-36α and IL-36γ were up-regulated in psoriatic skin lesions,\textsuperscript{35-37} and there was a strong correlation with expression
of other cytokines, including IL-17, IL-23, TNF-α, and IFN-γ. Following these observations, several groups developed genetic mouse models to investigate whether and what role the IL-36 family might play in the pathogenesis of psoriasis. Initially, Blumberg et al. demonstrated that transgenic mice overexpressing IL-36α in keratinocytes have a strong skin phenotype at birth that resolves at weaning. This phenotype is abrogated when transgenic mice are crossed with IL-36R- or AcP-deficient mice but strongly exacerbated when IL-36a transgenic mice are crossed with IL-36Ra-deficient mice. Phorbol ester treatment of mouse skin overexpressing IL-36α results in an inflammatory condition with macroscopic and histological similarities to human psoriasis. One of the most compelling lines of evidence for a role of IL-36 as a driver of skin inflammation was obtained by engrafting immunodeficient mice with human psoriasis skin. Treatment of these mice with an anti-human IL-36R-neutralizing mAb led to substantial normalization of skin pathology. Although the expression of IL-36Ra by IL-17-stimulated keratinocytes derived from patients with psoriasis does not differ from healthy controls, increased expression of the IL-36 cytokines correlates with Th17 cytokines in human psoriatic skin lesions and increased activation of MAPK and NF-kB. These results indicate that IL-36 may play an important role in psoriasis, as well as in other inflammatory diseases. In addition to psoriasis, the role of IL-36 has been studied in the pathogenesis of allergic contact dermatitis. Our group have demonstrated that gene expression of all 3 IL-36 agonists, but not IL-36Ra, was enhanced in ACD-involved skin. In addition ex vivo model showed that the addition of recombinant IL-36RA, in skin explants, causes the reduction of IL-36α, IL-36β e IL-36γ gene expression.
Pathogenesis of psoriasis

Psoriasis is a common chronic inflammatory skin disease with characteristic, sharply demarcated erythematous plaques restricted to distinct sites or disseminated over the skin surface. With a frequency of 2.5% in the Caucasian population, psoriasis is one of the most common chronic inflammatory skin diseases of man. Approximately 10–30% of patients with psoriasis develop psoriatic arthritis (PsA). Psoriasis can be provoked by nonspecific triggers such as mild trauma (scratching, piercings, and tattoos), sunburn, or chemical irritants. The major histological traits of psoriasis encompass acanthosis and hyperkeratosis of the epidermis, elongation of dermal capillary vessels and a lymphohistiocytic inflammation which are clinically reflected by erythema, infiltration and scaling. Underlying major pathogenic traits have been allocated to a heterogeneous (immuno)genetic background with a number of environmental and endogenous factors which result in a cellular and cytokine imbalance. Therefore, rather than viewing psoriasis as a disease caused by a single cell type or a single inflammatory cytokine, it is probably best to conceptualize its pathogenesis as linked to many interactive responses between infiltrating leucocytes, resident skin cells, and an array of proinflammatory cytokines, chemokines, and chemical mediators produced in the skin under regulation of the cellular immune system.

Psoriasis is mainly a dendritic cells (DCs) and T-cell-mediated disease with complex feedback loops from antigen-presenting cells, neutrophilic granulocytes, keratinocytes, vascular endothelial cells, and the cutaneous nervous system. Cross-talk between the innate and the adaptive immune system mediated by cytokines including TNFα, interferon γ, and interleukin 1 is a major research focus. TNFα is a pro-inflammatory cytokine that amplifies inflammation through several distinct pathways. TNFα is produced by a broad range of cell types including macrophages, lymphocytes, keratinocytes, and endothelial cells, and exerts its activities on several different cell types. TNFα induces secondary mediators and
adhesion molecules, all of which have been implicated in psoriatic disease. The clinical success of TNF-blocking agents is therefore not surprising.\textsuperscript{51} DCs, the most potent APCs (antigen-presenting cells), are sentinels of the immune system. In normal skin, DCs are found in the epidermis [LCs (Langerhans cells)] and dermis (myeloid and plasmacytoid DCs). LCs reside in the suprabasal layers of the epidermis in close contact with keratinocytes. After activation, LCs up-regulate chemokine receptors on their surface and migrate to skin-draining lymph nodes, where they present antigenic peptides they have encountered in the skin to naïve T-cells. The mobilization of LCs into draining lymph nodes in response to stimuli that normally induce migration [e.g. chemical allergen, TNF-\( \alpha \) and IL-1\( \beta \)] is largely absent in psoriasis.\textsuperscript{52} There are also increased numbers of plasmacytoid DCs in psoriatic skin compared with normal skin.\textsuperscript{53} These express TLR (Toll-like receptor) 9 and produce large amounts of IFN (interferon)-\( \alpha \) when activated with the microbicidal cathelicidin LL37 bound to self-DNA fragments released by stressed or dying cells in the skin. Plasmacytoid DC also express TLR8 and make IFN-\( \alpha \) when stimulated with self-RNA–LL37 complexes. LL37 can also bind self-RNA released by dying cells, and these complexes activate TLR7 in plasmacytoid DCs and, like self-DNA–LL37 complexes, trigger the secretion of IFN-\( \alpha \). In contrast with self-DNA–LL37 complexes, self-RNA–LL37 complexes can interact with TLR8 on classical myeloid DCs and promote their differentiation into mature DCs with secretion of TNF-\( \alpha \) and IL-6.\textsuperscript{52,53}

In psoriatic epidermis, keratinocytes proliferate and mature rapidly so that terminal differentiation, normally occurring in granular keratinocytes and then squamous corneocytes, is incomplete. Hence, squamous keratinocytes aberrantly retain intact nuclei (parakeratosis) and release few extracellular lipids that normally cement adhesions of corneocytes.\textsuperscript{44} Accordingly, poorly adherent stratum corneum is formed and this results in the characteristic scale or flakes of psoriasis lesions. Increased numbers of T lymphocytes are a
highly consistent finding in psoriasis biopsies. With immunohistochemical staining, T lymphocytes are found interspersed between keratinocytes throughout the epidermis and in somewhat larger quantities in the dermis.\textsuperscript{54-56}

Complex dysregulation of almost every cutaneous cell type, which includes proliferation and cytokine production by epidermal keratinocytes, is affected by the TNFα pathway and interleukin-23/Th17 axis pathway. Furthermore, antimicrobial peptides, cytokines and chemokines secreted by keratinocytes act as chemoattractants for infiltrating immune cells.\textsuperscript{57} Thus, a positive feedback loop exists between cells of the immune system and resident epithelial cells in psoriasis. Regulatory T cells affect the vascular endothelial growth factor (VEGF)-related angiogenic microenvironment\textsuperscript{58} and contribute to hallmark features of
Psoriasis such as epidermal hyperplasia. Psoriasis is a systemic inflammatory disorder that involves complex pathogenic interactions between the innate and adaptive immune system that can be targeted by innovative biological therapies.

Pathogenesis of allergic contact dermatitis

Allergic contact dermatitis (ACD) is a common inflammatory skin disease presenting with pruritic, eczematous lesions. ACD results from a T cell-mediated, delayed type hypersensitivity (DTH) reaction elicited by the contact of the skin with allergens (haptens). Contact allergens are low molecular weight (<500 Daltons) chemicals called haptens, which are able to penetrate the stratum corneum barrier of the skin. Haptens are not immunogenic by themselves, but they can be efficiently recognized by the immune system after binding to a skin protein carrier. Haptens may be naturally occurring substances such as urushiol found in the resin of poison ivy, synthetic compounds, dyes, fragrances, drugs, or heavy metal salts. Haptens activate Toll-like receptors (TLRs) and activate innate immunity. Keratinocytes are crucial for the development of ACD. They constitute the vast majority of cells in the epidermis and form the anatomic barrier of the skin, expressing most TLRs, and this allows them to respond to TLR4-triggering haptens, such as nickel. Keratinocytes are also a source of IL-10, an immunosuppressive cytokine that limits the extent of contact hypersensitivity. ACD is a type IV of DTH reaction that activates antigen-specific T cells in a sensitized individual. The sensitized T cells are primarily T-helper 1 (T\textsubscript{H}1) type. In the sensitization phase, innate immunity is activated through keratinocyte release of interleukin (IL)-1\textalpha, IL-1\textbeta, TNF\textalpha, granulocyte-macrophage colony-stimulating factor, and ILs-8 and -18. Langerhans and dermal dendritic cells uptake the allergen and migrate to the regional lymph nodes to activate antigen-specific T cells (ie, T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17, and regulatory T [Treg] cells). These T cells then proliferate and enter the circulation and
site of exposure. When re-exposed to the allergen, antigen-specific T cells are activated through the release of cytokines and induce an inflammatory process. Dermal dendritic cells, as opposed to epidermal Langerhans cells, play an important role in educating naïve T cells in the lymph node to become antigen-specific effector cells during cutaneous sensitization. The histopathologic findings are different in acute and chronic contact dermatitis and are dependent on the severity of the inflammatory reaction. The most common histologic feature is spongiosis, which results from intercellular edema. It is often limited to the lower epidermis but, if the reaction is severe, it may affect the upper layers.

Patch testing is the criterion standard in the diagnosis of ACD. Patch testing attempts to recreate, in vivo, an allergic reaction to nonirritating concentrations of an allergen that is suspended in a vehicle. Nickel is the most common cause of ACD in women in almost all
countries. The greater exposure of women to high-nickel content jewelry is a predisposing factor. Recent advances in knowledge of the mechanisms by which haptens can generate a specific T cell activation leading to ACD have reinforced the importance of hapten presentation by Langerhans cells to specific T cells.\textsuperscript{67} The induction of ACD depends on the production by epidermal cells, within minutes or hours following hapten application, of a rather specific pattern of cytokines. Indeed, previous study highlighted the complexity of the involvement of pro-inflammatory agonist and antagonistic members of the IL-1 family in the pathogenesis of ACD.\textsuperscript{43}

**Pathogenesis of polymorphic light eruption**

Polymorphic light eruption (PLE; "i.e., sun allergy") is an inflammatory skin condition considered as the most common idiopathic photodermatosis, with a prevalence of up to 21\% in temperate climates, particularly among young women.\textsuperscript{68-70} Although women are affected much more often,\textsuperscript{71,72} men also do develop the condition in significant numbers.\textsuperscript{73} PLE lesions usually appear in spring or early summer.\textsuperscript{70,74} Several morphological variants of PLE have been described, including papular, papulovesicular, plaque-type. The affected body sites are sun-exposed areas, particularly those that are normally covered during the winter such as the upper chest, the neck and the extensor aspects of the arms.\textsuperscript{75} Many individuals experience a hardening effect with prolonged sun exposure, occurring after repetitive exposures to UV radiation.\textsuperscript{74} This means that, as summer progresses, skin lesions are less likely to occur, or may be less severe than they were in early spring allowing prolonged sun exposure. Previous studies revealed that most PLE patients are sensitive to UVA, but lesions can also be induced with UVB alone, and some patients are sensitive to both waveband ranges.\textsuperscript{75,76} The pathogenesis of PLE remains unclear: a genetic susceptibility has been identified as well as environmental components.\textsuperscript{73,77} PLE is thought to be a delayed-type
hypersensitivity response (DTHR) against putative ultraviolet (UV)-induced de novo antigens, allowed to develop by a characteristic inadequate UV-induced immune-suppression.\textsuperscript{78,79} The delayed reaction time to UV radiation in PLE resembles that observed in immune responses mediated by CD4+ cells. A critical factor in the pathogenesis of PLE is the effect of UV light on skin components.\textsuperscript{79-81} It was hypothesized that in genetically predisposed subjects UV light induces a modification of certain skin molecules that renders them immunogenic. Supporting this hypothesis is the observation that cultured epidermal cells from PLE patients are capable of stimulating autologous peripheral blood mononuclear cells after exposure to high doses of UVA or UVB, suggesting that an immune sensitization against autologous UV light-modified skin antigens occurs in PLE.\textsuperscript{82} It is well known that the UV-induced suppression generates T cell-mediated responses in normal subjects involves the release of cytokines, particularly tumor necrosis factor (TNF\(\alpha\)), interleukin (IL)-4 and IL-10, and the migration of Langerhans cells (LCs) out of the epidermis.\textsuperscript{83} Conversely, PLE patients has a failure of UV-induced immunosuppression, which may favor the occurrence of autoimmunogenic skin rashes.\textsuperscript{78}
In healthy individuals, LCs disappear from the epidermis after UVB irradiation. IL-1β, TNF-α, and IL-18 release can modulate LC migration out of the skin. It was demonstrated that after a single high dose of UVB radiation much less LCs were depleted from the epidermis of PLE patients compared with healthy controls. Moreover, neutrophils infiltration and suppressor macrophages recruitment is significantly decreased in patients with PLE after UVB exposure, as well as T-regulatory limphocytes and related immunoregulatory factors after UVA1 irradiation. Because the incidence of UVB-susceptibility was significantly higher in skin cancer patients, if patients with PLE have a general increased resistence to UV-induced, these patients could be more resistant to UV
This is supported by the results of our recent study (by Lembo et al.) that investigated the link between PLE and skin cancer. Koelgen et al compared the expression of UVB-induced cytokines in the skin of normal individuals with PLE skin and their results showed that the skin of PLE patients contains lower levels of cytokines related to LC migration (IL-1, IL-18 and TNF-alpha).

The selection of the appropriate PLE treatment requires knowledge of the individual clinical course of the disease and depends on the frequency, duration and severity of the disease and the degree of lifestyle affection. Topical corticosteroids and occasionally oral antihistamines reduce the inflammation, alleviate itch and can shorten the duration of the eruption. Most PLE patients benefit from prophylactic treatment with phototherapy or photochemotherapy (hardening) to alleviate discomfort and lifestyle restrictions during the summer months or vacation periods in areas with high intensity sun exposure.

III Chapter

Experimental Design

Objective:
Our aim was to investigate on possible IL-36 involvement in some of most common inflammatory skin diseases such as, PSO, ACD and PLE.

Materials and methods

Study population
Our study population comprised: PLE patients, ACD patients, PSO patients and healthy controls. PLE patients were enrolled from the general dermatology or the photodermatology clinic of the University of Naples Federico II. The diagnosis of PLE was verified by patient's history, clinical symptoms, histological findings, specific laboratory studies (absence of
antinuclear antibodies etc.) and/or phototesting procedures. Each patient was required to answer a questionnaire designed from a previous version used for a former PLE study, aimed at establishing personal and family history of photodermatoses. Moreover, PLE severity of the last 12 months was measured through the PLE severity index (PLESI) questionnaire, on a 10 to 100 arbitrary scale. ACD patients were enrolled from the dermato-allergology center computer-based database of our Clinic. Inclusion criteria were age ≥18 years and the presence of at least one relevant positive reaction to patch test. The intensity of reactions were evaluated according to the guidelines of the Italian Society of Allergological, Occupational and Environmental Dermatology (SIDAPA). Results were recorded as negative, doubtful, and positive with 1+, 2+, 3+, respectively, for positive weak, moderate and strong reaction. PSO patients were recruited from the database of patients attending the psoriasis center of University of Naples Federico II, Naples, Italy. Inclusion criteria were diagnosis of moderate to severe psoriasis [Psoriasis Area and Severity Index (PASI) > 10] (with or without psoriatic arthritis) made by a dermatologist, disease duration of at least 6 months, topical and/or systemic treatment washout period of at least 3 weeks and age ≥18 years. Moreover, additional lesional skin biopsies were also performed in 10 out of 20 psoriatic patients after 16 weeks of treatments with anti-TNF-α (adalimumab) treatment. Control skin was obtained from surgical remnants of healthy donors undergoing abdominoplastic, with no history of photodermatosis, skin cancer or on-going inflammatory skin disease. The study was conducted according to the Declaration of Helsinki principles. The local ethical committee approved the study, and each participant gave written informed consent before entering the study.

Phototesting procedures

In all PLE patients, both UVA and UVB minimal erythema dose (MED) were obtained from untanned areas of the lower back by means of the Solar Simulator Multiport 601 (Solar Light
Company, Philadelphia, PA, USA). As indicated by the European Dermatology Guideline for the photodermatoses, provocative phototests with UVA and UVB were performed on a healthy and untanned area of the back or arms with the following procedure: a 5-10 cm² selected area was divided into three parts: the first one was irradiated with UVA only (metal halide lamp equipped with H1 and H2 filters, Dr Honle, Munich, Germany); the second one with UVB radiation only (TL12 fluorescent lamps, Philips, Amsterdam, the Netherlands) and the third one was irradiated with both UVA and UVB wavelengths. Irradiance measurement was performed before each irradiation session using an Il-1700 radiometer (Technology Drive, Peabody, Massachusetts, USA). The total dose given daily over 3–5 consecutive days was of 50-120 m J/cm² UVB (corresponding to 0.75–1.5 of the calculated UVB MED) and 30 J/cm² of UVA. The results were read at 2, 5, 7 up to 10 days. Phototests resulted negative in 64.7% (n=11/17) and positive in 35.3% (n=6/17) of the examined patients. In particular: palpable erythema after UVA was appreciated in 11.8% (n=2/17) with similar reaction observed also adding UVB to the UVA wavelength, but not after specific UVB irradiation. Conversely UVB reactivity was observed in 17.6% (n=3/17) with similar reaction also obtained after stimulation with UVB and UVA. One patient (5.9%) reacted only to the mixed irradiation (UVB+ UVA).

Patients biopsies, RNA extraction, cDNA synthesis and real-time polymerase chain reaction (rt-PCR)
Skin biopsies from PLE, ACD, PSO and healthy controls were collected. In the PLE and PSO patients, the biopsies were taken from spontaneous lesion observed during clinical assessment. Conversely, in the ACD patients, skin samples were taken from the positive reaction to the patch test. RNA was extracted (RNeasy Mini Protocol, Qiagen Valencia,CA) and cDNA was prepared (Transcriptor High Fidelity cDNA Synthesis, Roche, Indianapolis,
IN) according to the manufacturer’s instructions. rt-PCR (LightCycler, Roche, Indianapolis, IN) was used to analyze the levels of expression of 18S, IL-36Ra, IL-36α, IL-36β, IL-36γ. Each PCR was performed 2 times, each time in triplicate. PCR protocol and product quantification for 18S ribosomal RNA were performed as reported previously.28 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, 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 CT method. Results obtained from each PCR were pooled and statistically analyzed.

**In vivo expression of IL-36 in the skin**

Skin biopsies (3 mm) were collected from involved sites of subjects affected by PLE (n=17), psoriasis (n=20), ACD (n=10) as well as from normal skin of healthy control subjects (n=15) for *in vivo* mRNA examination through real-time PCR.

**In vivo expression of IL-36 in psoriasis subjects before and after anti-TNF-α treatment**

Skin biopsies (3 mm) from involved sites of 10 out of 20 psoriatic patients were collected before (week 0) and after 16 weeks of anti-TNF-α (adalimumab) treatment for *in vivo* mRNA examination through real-time PCR. Adalimumab was administered subcutaneously 80 mg at week 0 (baseline) and successively 40 mg every other week starting from week 1 and up to week 16.

**Immunohistochemical detection of IL-36 family members**

The immunohistochemical detection of IL-36 family members was carried out on the skin biopsies from the lesions of PLE, ACD, PSO patients and healthy 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. Some slices were initially stained with haematoxylin (Fisher Scientific, Pittsburgh, PA, U.S.A.) and eosin (Shandon, Pittsburg, PA, U.S.A.) for morphological evaluation. Thereafter, staining for IL-36α (15 μg/ml R&D System Inc, Minneapolis, MN), IL-36β (5 μg/ml R&D System Inc, Minneapolis, MN), IL-36γ (15 μg/ml R&D System Inc, Minneapolis, MN) and IL-36Ra (5 μg/ml R&D System Inc, Minneapolis, MN) was performed, incubating tissue sections at 4°C overnight. In parallel, other sections were incubated with specific isotype control antibodies (Mouse IgG1-2B Isotype Control, Goat IgG Control, R&D System Inc, Minneapolis, MN) used at the same concentration as the corresponding primary antibody. All 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 grading the staining intensity, the T-MARKER (http://comp-path.inf.ethz.ch) image analysis software was used according to the standardized protocol. Mean intensity values of all analyzed interleukins were calculated for each patient and control.

**IL-36γ serum levels in psoriasis, ACD and healthy control subjects**

Blood samples were taken from the subjects affected by psoriasis (n=20) and ACD (n=10) as well as healthy control subjects (n=10) for the determination of IL-36γ concentrations by single enzyme-linked immunosorbent assay (ELISA). Commercially available kit (Uscn, Life Science, Inc.) was used according to the manufacturer’s instructions.
Supernatants collected from healthy skin organ culture, performed as previously described, stimulated with TNF-α (20ng/ml) for 24h were used as positive controls. These supernatants were checked for the presence of IL-36γ by western blot analysis too and a significant increase was detected after TNF-α treatment.

**IL-36γ serum levels in PLE patients**

Blood samples were collected by venepuncture, into plain collection tubes for serum, from PLE patients and controls. Extracted serum proteins were loaded (20 µg of proteins) on the 10% Bis-Tris NuPage denaturing gel (Invitrogen Life Technologies, Carlsbad, CA) for electrophoresis. Gels were transferred to nitrocellulose membranes and were probed with IL-36 γ monoclonal antibody (R&D System Inc, Minneapolis, MN); detection performed with WesternDot™ 625 western blot detection kit (Invitrogen Life Technologies, Grand Island, NY). The enhanced fluorescent bands were assessed using Gel-Doc densitometer and Quantity-One software (Bio-Rad, Hercules, CA).

**In vitro expression of IL-36γ in peripheral blood mononuclear cells (PBMC)**

PBMC were isolated from subjects affected by psoriasis (n=5) and ACD (n=5) as well as healthy control subjects (n=5) by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Cell number and viability were determined using Trypan blue test. mRNA was extracted from 1x10⁶ cells for examination through real-time PCR.

**In vitro expression of IL-36γ in stimulated PBMC**

PBMC isolated from 5 healthy controls, 5 psoriatic and 5 nickel-ACD (#1-5) patients were cultured in Roswell Park Memorial Institute (RPMI; GIBCO) medium, supplemented with antibiotics (100 IU/ml penicillin G and 100 lg/ml streptomycin; GIBCO) and 10% FBS (GIBCO), at a concentration of 2 x 10⁶ in 12-well plates. They were stimulated with 5µg/ml of concanavalin A (ConcA) (Sigma-Aldrich, Oakville, ON, Canada) or 100ng/ml of toxic shock syndrome toxin (TSST-1) (Sigma-Aldrich, Oakville, ON, Canada) or 10⁻⁴M of NiSO₄.
They were incubated for 24 hours at 5% CO2 at 37°C. mRNA was extracted for examination through real-time PCR.

*Ex vivo expression of IL-36γ in ACD non lesional skin assay*

4 skin biopsies (3 mm each) from each of 5 nickel-ACD (#1-5) patients and 4 skin biopsies (3mm each) from each of 5 healthy subjects who had undergone plastic surgery were performed to execute *ex vivo* ACD assays. Skin biopsies of nickel-ACD patients were taken from uninvolved sites. 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). In this way the epidermis faces upwards at the liquid-air interface whereas the dermis is suspended in the culture medium. The 4 skin biopsies (both of nickel-ACD and healthy subjects) were treated as follows: i) 1 skin biopsy was cultured only with medium and used as control, ii) 1 skin biopsy was exposed to the allergen that had previously elicited the positive patch test reaction (NiSO₄), iii) 1 skin biopsy was exposed to NiSO₄ and contemporarily PBMC isolated from the same subjects were added to the multiwell (not into the transwell filter), iv) 1 skin biopsy was cultured with medium and contemporarily PBMC isolated from the same subjects were added to the multiwell (not into the transwell filter). This system was incubated for 72 hours at 5% CO2 at 37°C. The quantity of NiSO₄ (5% in petrolatum) used for these assays (30 mg) was the same used for the patch tests. After 72 hrs mRNA was extracted from PBMC, separately collected, and from cultured skin biopsies for examination through real-time PCR.
Statistical analyses

Data that passed the normality test were analyzed with two-tailed, t test, otherwise Wilcoxon test was used to calculate statistical differences. P values <0.05 were considered statistically significant. *p<0.05; **p<0.01; ***p<0.001. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc, La Jolla, CA).

Results

IL-36 cytokines gene expression in lesional skin

As showed in Fig.1, gene expression of IL-36α and IL-36γ was significantly increased in PLE skin compared to healthy controls (HS). Conversely IL-36β, IL-36Ra and IL-33 did not increase in PLE skin. In ACD as well as PSO, all analyzed interleukins were significantly increased, except for IL-36 receptor antagonist (IL-36Ra) in ACD. IL-36γ was expressed at higher level in PLE skin rather than PSO and ACD skin.
Figure 1. IL-36 expression in lesional skin: mRNA fold expression of IL-36 (α, β, γ) and IL-36Ra (Ra) in healthy control (HS), PLE, PSO and ACD skin biopsies. PCR values are expressed as fold increase expression normalized to the housekeeping gene 18S respect to control skin from normal donors. Data are displayed as mean± standard deviation (SD); *p<0.05; **p<0.01; ***p<0.001

Immunohistochemical detection of IL-36 cytokines

In addition, we also evaluated IL-36 cytokines at protein level by IHC in PLE, PSO and ACD patients compared to healthy skin samples. IL-36 agonists resulted enhanced in all 3 analyzed diseases. In particular the increase was more marked and diffuse through the epidermal layers in ACD and PSO samples, whereas it resulted moderate and mainly localized to granular and spinous layers in PLE. Results of the TMARKER image analysis of staining intensity confirmed immunohistochemical data.

<table>
<thead>
<tr>
<th></th>
<th>IL-36Ra (Mean ± SEM)</th>
<th>IL-36α (Mean ± SEM)</th>
<th>IL-36β (Mean ± SEM)</th>
<th>IL-36γ (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLE</td>
<td>4.2 ± 0.84</td>
<td>48.0 ± 1.08</td>
<td>41.0 ± 3.82</td>
<td>74.0 ± 4.12</td>
</tr>
<tr>
<td>PSO</td>
<td>4.5 ± 1.0</td>
<td>57.3 ± 1.86</td>
<td>42.31 ± 2.56</td>
<td>74.8 ± 2.98</td>
</tr>
<tr>
<td>ACD</td>
<td>5.1 ± 0.41</td>
<td>55.0 ± 2.08</td>
<td>45.0 ± 1.23</td>
<td>72.7 ± 3.15</td>
</tr>
<tr>
<td>HS</td>
<td>1.72 ± 0.17</td>
<td>3.33 ± 1.84</td>
<td>16.35 ± 0.32</td>
<td>4.51 ± 0.13</td>
</tr>
</tbody>
</table>

Table 1. TMARKER results: Immunohistochemistry staining intensities. Statistical significance calculated over staining in the control skin. SEM: standard error of the mean.
Figure 2. IL-36 protein expression in lesional skin: Immunohistochemical detection of IL-36 (α, β, γ) and IL-36Ra in skin biopsies of PLE, PSO, ACD patients and healthy donors (HS). Each picture is representative of data from 15 different patients affected by PLE, ACD and 10 normal donors.

Anti-TNF-α treatment decreases IL-36 gene expression in psoriatic skin

An efficacious anti-TNF-α treatment is able to downregulate significantly (*p<0.05; **p<0.01) all the pro- and anti-inflammatory members of IL-36 subfamily, except for IL-36β.
Figure 3. Anti-TNF-α treatment modulates IL-36 gene expression: mRNA fold expression of IL-36 (α, β, γ) and IL-36Ra (Ra) in lesional skin of 10 psoriasis patients before and after 16 weeks of anti-TNF-α treatment. PCR values are expressed as fold increase expression normalized to the housekeeping gene 18S respect to control skin from normal donors. Data are displayed as mean± standard deviation (SD); *p<0.05; **p<0.01; ***p<0.001

IL-36γ serum levels in psoriasis, ACD and healthy control subjects

Next, we analyzed IL-36γ serum levels in ACD and PSO patients and no significant differences were found, even when comparing with healthy controls. This result was confirmed by the presence of a positive control checked by western blot analysis too. Supernatants collected from healthy skin organ culture, performed as previously described, stimulated with TNF-α (20ng/ml) for 24h were used as positive controls. These supernatants were checked for the presence of IL-36γ by western blot analysis too and a significant increase was detected after TNF-α treatment.
Figure 4. IL-36γ serum levels: HC (n=10), PSO (n=10) and ACD (n=10) subjects. Supernatants from healthy skin organ culture treated with TNF-α (20ng/ml) for 24h are used as positive controls (CTRL+). Data are displayed as mean± standard deviation (SD);

Western blot analysis of IL-36γ

Since we have detected the highest increase among all analyzed conditions for IL-36γ in PLE, we sought to better explore this cytokine also at serum level. A significant enhance was found compared to healthy donors, in contrast to other skin inflammatory conditions as above showed.
Figure 5. IL-36γ protein levels in PLE patients: A) Representative western blot image analysis of IL-36γ in PLE patients and healthy controls. B) Densitometry is plotted as adjusted volume density (intensity mm²)

IL-36γ expression in peripheral blood mononuclear cells (PBMC)

We checked for IL-36γ gene expression in circulating PBMC isolated from psoriatic and ACD patients as well as healthy controls, with no significant differences found (Fig.6A). Additionally, in order to further elicit a response, PBMC isolated from healthy subjects, psoriatic and nickel-ACD patients were exposed in vitro to concanavalin A (ConcA), toxic shock syndrome toxin (TSST-1) and to nickel sulphate (NiSO4). Only the stimulation with NiSO4 in ACD PBMC was able to induce a significant increase (*p<0.05) of IL-36γ respect to healthy controls (Fig.6B).
**Figure 6. In vitro expression IL-36γ**: A) IL-36γ gene expression in PBMC of HC (n=5), PSO (n=5) and ACD (n=5) subjects. B) IL-36γ gene expression in stimulated PBMC with concanavalin A (ConcA; 5µg/ml), toxic shock syndrome toxin (TSST-1; 100ng/ml) and nickel (NiSO₄; 10-4M) for 24 hours.

**Ex vivo expression of IL-36γ in ACD non lesional skin assay**

At this point, we wanted to highlight IL-36γ involvement in ACD and *ex vivo* ACD non lesional skin assay was performed. IL-36γ expression was significantly augmented in uninvolved ACD skin when treated with the allergen (NiSO₄), but an even more enhanced increase was obtained in uninvolved ACD skin when PBMC were added to the system.
Figure 7. *Ex vivo non lesional skin assay*: IL-36γ gene expression in healthy skin (HS; n=5) and in non lesional (NLes) nickel-ACD skin (n=5) stimulated with 30mg of NiSO₄ (5% in petrolatum). PBMC isolated from the same subjects were incubated in the system with or without NiSO₄.
IV CHAPTER

Discussion

Over recent years much has been learned on IL-36 cytokines about their important functions in the regulation of immune response and, especially, on their involvement in many inflammatory conditions.\textsuperscript{21,22,27,36} In this study, we wanted to elucidate the role of these mediators in some of the most common skin inflammatory diseases, evaluating their levels in psoriasis, ACD and PLE. We found that the increase of all 3 agonist members was more evident respect to receptor antagonist. Recently, D’ Erme et al. have reported IL-36γ as having a highly specific positive predictive diagnostic value for psoriasis.\textsuperscript{90} This is in part true, also according to our data, even though we strongly believe that IL-36 agonists are involved not only in psoriasis, but also in ACD as well as PLE. We shown that IL-36α, IL-36β, and IL-36γ are enhanced in ACD and PSO skin too.\textsuperscript{36,43} We speculate that they might represent markers of skin inflammation in general, and it seems weakly reliable that IL-36γ alone might constitute a specific psoriasis marker. We have performed a comparison for skin IL-36 gene expression in the 3 examined disorders was performed, with a significant increase, respect to healthy subjects, for all members, except for IL-36 receptor antagonist (IL-36Ra) in PLE as well as ACD. These results were confirmed at protein level by immunohistochemical analysis of skin biopsies. Indeed, IL-36 members resulted enhanced in 3 all analyzed diseases compared to healthy skin, except for IL-36Ra. This last one is the anti-inflammatory member of IL-36 sub-family and the lack of the natural antagonist in ACD let us to hypothesize that the activity of IL-36α, IL-36β and IL-36γ is ‘unopposed’ and therefore that these cytokines can drive skin inflammation.\textsuperscript{43} Nevertheless, we found that IL-36γ was expressed at higher level in PLE as well as psoriatic skin rather than ACD skin. This finding was in agreement with Quaranta et al.,\textsuperscript{91} who found a significant increase of IL-36γ both in psoriatic and eczematous skin, with a main up-regulation in psoriasis. The impact of
an anti-TNF-α treatment on psoriatic skin levels of IL-36 (α, β and γ) and IL-36Ra was evaluated, with significant decrease for all analyzed interleukins, except for IL-36β. This data reflects the fact that an efficacious treatment is able to downregulate all the pro- and anti-inflammatory members of IL-36 subfamily, indiscriminately. Next, we analyzed IL-36γ serum levels in 2 categories of patients (PSO; ACD) and no significant differences were found, even when comparing with healthy controls; this result was confirmed by the presence of a positive control checked by western blot analysis too. Furthermore, we assessed serum IL-36γ levels by western blot in PLE patients too. Conversely, we found an increase of IL-36γ circulating cytokine in PLE compared with healthy controls. This data could confirm the presence of a systemic inflammation of these patients. Wolf et al. reported that PLE patients had a significantly higher baseline plasma level of pro-inflammatory cytokines. Next, we analyzed IL-36γ gene expression in circulating peripheral blood mononuclear cells (PBMC) isolated from psoriatic and ACD patients as well as healthy controls, with no significant differences found. Moreover, in order to further elicit a response, PBMC isolated from healthy subjects, psoriatic and nickel-ACD patients were exposed in vitro to concanavalin A (ConcA), toxic shock syndrome toxin (TSST-1) and to nickel sulphate (NiSO₄). Only the stimulation with NiSO₄ in ACD PBMC was able to induce a significant increase of IL-36γ respect to healthy controls, suggesting that this IL might be involved in ACD too. At this point, we wanted to highlight IL-36γ involvement in ACD and ex vivo ACD non lesional skin assay was performed. IL-36γ gene expression was significantly augmented in uninvolved ACD skin when treated with the allergen (NiSO₄), but an even more enhanced increase was obtained in uninvolved ACD skin when PBMC were added to the system. In order to deeply comprehend the contribution given by PBMC, we compared IL-36γ gene expression as well as of its known inducers (TNF-α and IL-1α) in nickel-ACD PBMC treated in vitro with NiSO₄ and nickel-ACD PBMC incubated in ex vivo ACD non lesional skin assay.
significant increase was detected for all ILs in PBMC that had been incubated in the skin system.

Indeed, it is well known that skin inflammation results from the multipartite interactions of, at least, keratinocytes (KCs), T cells and antigen presenting cells (APCs). In this scenario KCs are the major source of IL-36γ which contributes to skin inflammation by acting on KCs, APCs and indirectly upon T cells driving tissue infiltration, cell death and proliferation\textsuperscript{25,92}; Tortola et al., 2012). We speculate that in \textit{ex vivo} ACD non lesional skin system, IL-36γ produced by KCs promotes skin T cells infiltration amplifying cutaneous damage with an increase in IL-36γ. The expression of IL-36γ is highly complex and it may have distinct functions dependent upon the context in which it is expressed. It is reported that IL-36γ acts as an alarmin, being up-regulated by toll-like receptor ligand expressed in response to the innate immune system activation and having a central position in pro-inflammatory pathways at the interface between innate and adaptive immunity.\textsuperscript{93} Furthermore, IL-36γ induces the production of several proinflammatory cytokines, including IL-12, IL-6, TNF-α, and IL-23,\textsuperscript{41} involved both in psoriasis and ACD. All these evidences support our hypothesis that IL-36γ could represent a marker for skin inflammation and not a specific one for psoriasis.
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