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XXV Ciclo

*Interleukin-1 family members are enhanced in psoriasis
and suppressed by vitamin D and retinoic acid*

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Introduction

Psoriasis is an immune-mediated skin disease that affects 1–3% of the population worldwide, with an equal sex distribution (Lowes MA *et al.*, 2007). The common form of the disease, termed ‘plaque psoriasis vulgaris’, is observed in more than 80% of patients and is characterized by erythematous scaly plaques, typically on elbows, knees, scalp and buttocks. Approximately 10–30% of patients with psoriasis develop psoriatic arthritis (Nestle FO *et al.*, 2004). Histological examination of psoriatic plaques reveals keratinocyte hyperproliferation with parakeratosis and elongation or rete ridges, increased angiogenesis and dermal infiltration of inflammatory cells, including T cells, neutrophils, macrophages and DCs (dendritic cells). The interleukin (IL)-1 family comprises 11 members that play an important role in immune regulation and inflammatory process. Recent studies showed that IL-1 family contributes to psoriasis pathogenesis (Balato A, *et al.*, 2013, Carrier Y *et al.*, 2011 Johnston A *et al.*, 2011). Because of the wide range of clinical manifestations and the chronic nature of psoriasis, therapeutic approaches for these patients should be individualized taking into account the extent of skin lesions, the concomitant presence of arthritis and other co-morbidities. Vitamin D (vitD) exerts pluripotent effects on adaptive immune functions such as T cell activation and maturation of DCs. In addition, vitD has been suggested to increase innate immunity in skin and to enable efficient antimicrobial defense at epithelial surfaces (Schauber J *et al.*, 2008). In particular, VitD and analogs have been shown to suppress TNF (tumour necrosis factor)- α -induced IL-1 α in human keratinocytes (KCs) (Kong J *et al.*, 2006). Moreover, retinoids exert complex effects on the immune system, having anti-inflammatory effects in chronic dermatological diseases (Gross V *et al.*, 1993, Bollag W 1983). In the present study, we

investigated IL-1 family members in psoriasis and the effects of vitD and retinoic acid (RA) on these members.

Chapter I

Immunopathogenesis of psoriasis

Genetic basis of psoriasis

Psoriasis is a complex multifactorial disease, where various environmental triggers (e.g. trauma, stress, infections and drugs) promote, in genetically predisposed individuals, the activation of an exaggerated and poorly controlled immuno-inflammatory response in the skin. The incidence of psoriasis is greater in relatives of patients than among the general population, even though segregation analyses show no clear pattern of inheritance. Disease concordance rates are much higher in monozygotic twins (65–72%) than in dizygotic twins (15–30%). Genome-wide linkage scans have been conducted in the search for genes predisposing to familial psoriasis (Nair R. P *et al.*, 2009) These have led to the identification of several putative susceptible loci on different chromosomes, but very few have been replicated in independent family sets. The only locus consistently identified is on the short arm of chromosome 6, named *PSORS1* (psoriasis susceptibility 1) (Capon F *et al.*, 2004). Three genes contained within this region are associated with psoriasis, namely *HLA-Cw6*, *CCHCR1* (coiled-coil α -helical rod protein) and *CDSN* (corneodesmosin). *HLA-Cw*0602*, a variant of *HLA C*, encodes a class I MHC protein and is associated with early-onset chronic plaque psoriasis and with early-onset chronic plaque psoriasis and with guttate psoriasis, an acute-onset childhood form of the disease triggered by streptococcal infections. *CCHCR1* encodes coiled-coil α -helical rod protein 1, which is highly overexpressed in psoriatic skin, and is supposed to be a regulator of keratinocyte proliferation. *CDSN* encodes corneodesmosin, a late differentiation epidermal glycoprotein overexpressed in psoriatic skin and putatively involved in

keratinocyte adhesion. *PSORS1* accounts for 35–50% of the heritability of the diseases, but does not explain the entire genetic predisposition. Other susceptibility loci identified include genes expressed in keratinocytes, immune cells and involved in skin barrier function and immune responses against pathogens.. Combinations of functional changes associated with the variants of these genes could favour the early steps of the plaque-inducing immune response (Fig. 1).

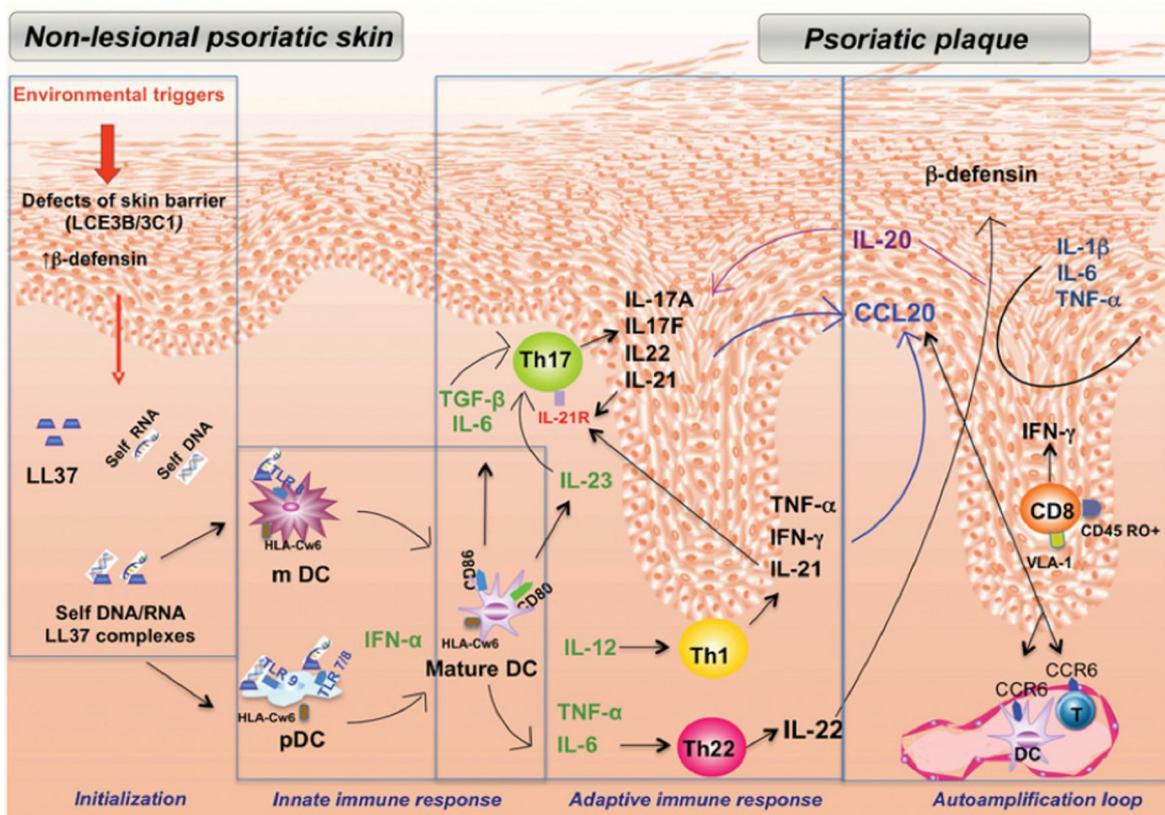


Fig.1 Main actors of psoriasis pathogenesis.

Cellular basis of psoriasis

Rather than viewing psoriasis as a disease caused by a single cell type or a single inflammatory cytokine, it is probably best to conceptualise disease 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. 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- α and IL- 1β] is largely absent in psoriasis (Lowes MA *et al.*, 2007). These findings raise the possibility that LCs retained within the epidermis present antigens locally and exacerbate the ongoing inflammatory reaction. In the inflamed dermis of psoriatic patients, there is a marked increase in myeloid CD11c+ DCs. These cells, probably derived from circulating DC precursors, migrate into the skin in response to chemotactic signals and synthesize high levels of pro-inflammatory cytokines (e.g. IL-12 and IL-23) [Zaba LC *et al.*, 2007]. The dermal CD11c+ DCs which make TNF- α and iNOS (inducible nitric oxide synthase) (TIP-DCs) are thought to be the human equivalent of a similar DC subset which is needed for the clearance of some pathogens in mice. There are also increased numbers of plasmacytoid DCs in psoriatic skin compared with normal skin. These express TLR (Toll-like receptor) 9 and produce large amounts of IFN (interferon)- α when activated with the microbicidal cathelicidin LL37 bound to self-DNA fragments released by stressed or dying cells in the skin (Figure 1) (Lande R *et al.*, 2007). Plasmacytoid DC also express TLR8 and make IFN- α when stimulated with self-RNA-LL37 complexes (Figure 1). 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- α . 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- α and IL-6 (Figure 1). Consistently, in mice, topical applications of the TLR7/TLR8 ligand imiquimod induce psoriasis-like skin inflammation. KCs can be viewed as an integral part of the skin-resident immune system, because they may act as APCs, produce innate immune mediators, and contribute to the skin homing and local activation of immune cells. KCs express TLRs and respond to microbial stimuli by producing large amounts of cytokines (e.g. TNF- α , IL-1 α , IL-6 and IL-18), chemotactic chemokines [e.g. IL-8 and CCL20 (CC chemokine ligand 20)] and antimicrobial peptides [e.g. HBD (human β -defensin)-2, HBD-3 and LL37]. Moreover, the psoriatic plaque is characterized by a marked infiltration of activated CD4⁺ and CD8⁺ T-cells. CD4⁺ T-cells infiltrate mainly the dermis, whereas CD8⁺ T-cells are present in the epidermis. Th (helper) 1 and Th2 are the best understood effector CD4⁺ T-cells formed during immune responses. Th1 cells produce IFN- γ and TNF- α , and mediate immune responses against intracellular bacteria, viruses and tumour cells. Th2 cells make mostly IL-4, IL-5 and IL-13, and stimulate humoral responses against extracellular parasites. Another subset of Th cells, termed Th17 cells, which are predominantly CD161-expressing cells, secrete IL-17A and IL-17F, and are involved in the activation of neutrophils and immunity to bacteria and fungi. TGF- β 1 (transforming growth factor- β 1) concentration and the concomitant presence of at least one proinflammatory cytokine are key factors in human Th17 differentiation.. These cells, together with Th1 cells, (Robinson DS *et al.*, 2002), appear to lie at the very heart of the pathogenesis of psoriasis (Zaba *et al.*, 2007). Indeed, IL-17 produced by Th17 cells was shown to promote the production of IL-6, IL-8, GM-CSF (granulocyte/macrophage colony-stimulating factor) and ICAM-1 (intercellular adhesion molecule-1) in KCs, synergizing with IFN- γ . They can also produce IL-21, IL-22 and IL-26. IL-21 acts on Th17 cells to amplify its own synthesis. IL-21 also up-regulates IL-23R expression on T-cells, thus making Th17 cells responsive to IL-23. Altogether, these

events generate a positive-feedback loop that helps amplify the Th17 lineage. More recently, it has been shown that the IL-22 expression profile may differ from that of IL-17A, and that IL-22-producing T-cells, termed Th22 cells, could represent a T-cell subset that is distinct from typical Th17 cells (Zheng Y *et al.*, 2007). Traditionally, psoriasis has been classified as a Th1-associated disease, because T-cells infiltrating the lesional skin of psoriatic patients produce high levels of IFN- γ . IL-12, the major Th1-inducing factor in humans, is also highly expressed (Figure 1). However, in the psoriatic plaque, there is also elevated synthesis of Th17-related cytokines, such as IL-17A, IL-17F, IL-21 and IL-22 (Figure 1), as well as enhanced production of IL-23, a heterodimeric cytokine composed of IL-23p19 and IL-12p40 subunits which amplifies Th17 cell responses and causes psoriasis lesions when administered intradermally to mice. A functional role of Th17 cells in psoriasis is suggested by the demonstration that both IL-21 and IL-22 induce keratinocyte hyperplasia and that Th17 cytokine levels decrease during successful anti-TNF- α treatment. IL-22 also triggers the production of antimicrobial peptides and expression of genes involved in epidermal differentiation and survival. Studies in mice have shown that IL-22 induces keratinocyte hyperplasia and acanthosis, and that some biological effects of IL-22 are amplified by TNF- α , as a result of the ability of TNF- α to enhance IL-22 receptor expression (Zheng Y *et al.*, 2007). Both Th1 and Th17 cytokines induce keratinocytes to produce CCL20, a chemoattractant for CCR6-expressing DCs and T-cells (Figure 1), thus providing a positive feedback loop that sustains the accumulation of these cells in the psoriatic skin. IL-17A and IL-22 are powerful inducers of IL-20 by DCs and proliferating keratinocytes (Figure 1). IL-20 is highly produced in psoriasis, and its overexpression in transgenic mice causes epidermal thickening. Interestingly, the skin alterations in IL-20-transgenic mice occur without immune cell infiltration, suggesting that IL-20 is a downstream mediator in the psoriasis-associated immuno-inflammatory cascade. Therefore the IL-23/Th17 pathway appears to be central in the pathogenesis of

psoriasis, orchestrating both the induction and maintenance of skin inflammatory response by regulating the secretion of inflammatory cytokines and chemoattractants, and the proliferative response of psoriatic KCs through the production of mitogenic cytokines (IL-22 and IL-21).

Chapter II

The IL-1 family: regulators of immunity

Members of the IL-1 family

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 (Sims *et al.*, 2010). 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. The IL-1 family comprises 11 members: IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra), IL-18, IL-33 and IL-36 cytokines, IL-36 α , IL-36 β and IL-36 γ , previously designated IL-1F6, IL-1F8, and IL-1F9, respectively (Figure 2). They probably arose from the duplication of a common ancestral gene, as they possess a highly conserved gene structure, including identical positioning of certain introns and a more modest preservation of key amino acid sequences that allow the folding of each protein into a 12-stranded β -barrel⁷. All the genes encoding IL-1 family members, except IL-18 and IL-33, are clustered in a 400 kb region of human chromosome 2 (Dinarello C *et al.*, 2010). The mechanism of secretion of the other IL-1 family members remains unknown.

IL-1 β is mainly produced by monocytes and macrophages, whereas IL-1 α expression is more widespread; for example, it is highly expressed by keratinocytes and endothelial cells, IL-1Ra is mainly expressed by cells that produce IL-1 (for example, monocytes, B cells and certain basal epithelial cells), and its primary role may be to prevent excessive autocrine activation of the IL-1 signalling pathway. IL-18 is expressed by macrophages and DCs as well as epithelial cells, such as keratinocytes, whereas the production of IL-1 typically requires an inducing stimulus, pro-IL-18 is constitutively expressed, but needs processing by caspase 1 to be activated. IL-33 is the most recently discovered IL-1 family member and, in contrast to other family members, is not typically expressed by haematopoietic cells but is abundantly expressed in many tissues. It was originally characterized as a nuclear protein prominent in high endothelial venules, IL-33 has an N-terminal pro-domain that contains a helix–turn–helix motif that is typical of DNA-binding proteins. In its nuclear (uncleaved) form, it interacts with histones 2A and 2B in heterochromatin and by promoting chromatin compaction functions as a transcriptional repressor. It is currently not clear how IL-33 is released from cells and whether a cleaved or full-length form is the active cytokine *in vivo*. IL-36Ra and IL-36 cytokines are abundantly expressed in skin and a few other tissues unique to each molecule, and their expression can be strongly induced in monocytes³⁴ These cytokines will provide insight into the regulation of the activity of the cytokines and potentially offer a point of therapeutic intervention.

IL-1 family members signal through a group of closely related receptors (Figure. 2), and many of the encoding genes are also clustered in a short region of human chromosome 2. The response is initiated when the ligand binds to its primary receptor subunit; IL-1 α and IL-1 β bind to IL-1 receptor (IL-1R1), whereas IL-1 receptor antagonist (IL-1Ra) has an antagonistic effect. Recently, pro-inflammatory properties have been reported also for IL-36 cytokines (Carrier Y et al., 2011). All IL-36 bind IL-1-receptor-like 2 (IL-1Rp2), now

named IL-36 receptor (IL-36R), whereas IL-36Ra acts as receptor antagonist, IL-33 binds to interleukin 1 receptor-like 1 (IL-1RL1) and a proinflammatory activity is started too. IL-37 produces anti-inflammatory effects, whereas IL-38, binding to IL-36R, has similar effects to IL-36Ra (Sharma S *et al.*, 2008, Nold MF *et al.*, 2010, Van der Veerdonk FL *et al.*, 2012), Binding of the ligand allows the recruitment of a second receptor subunit and the recruitment of myeloid differentiation primary response protein 88 (MYD88), IL-1R associated kinase 4 (IRAK4), TNFR-associated factor 6 (TRAF6) and other signalling intermediates. The ensuing biological response typically involves the activation of the nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways (Towne JE *et al.*, 2004)

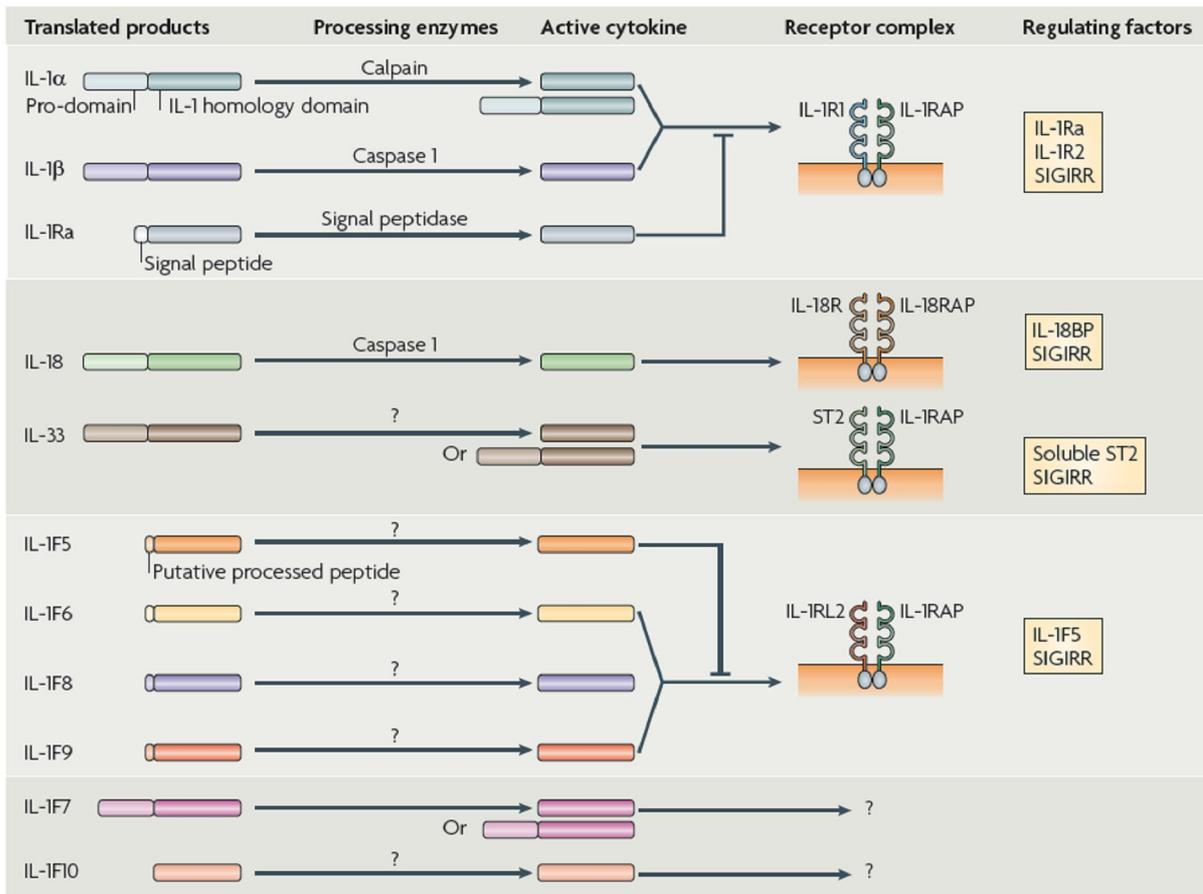


Fig.2 Interleukin-1 family members, processing, receptors and regulation.

Effects on innate immune cells

The ability of most IL-1 family members to directly activate or otherwise enhance the effector functions of innate immune cells suggests they are endogenous activators of innate inflammatory responses. Eosinophils are particularly influenced by IL-33, probably owing to their abundant expression of ST2. IL-33 acts directly on eosinophils to enhance their survival and adhesive properties, as well as their production of superoxide and CXC-chemokine ligand 8. Mast cells mediate innate allergic responses and are potently influenced by IL-1 family signalling. IL-1 and IL-18 enhance the secretion of cytokines such as IL-3, IL-5, IL-6, IL-13 and TNF- α by mast cells, but only in the presence of an additional signal, such as IgE or IL-3. By contrast, IL-33 stimulates cytokine production by mast cells in the absence of additional signals. IL-33 also increases mast cell maturation and survival and enhances the effects of other mast cell activation stimuli. IL-1 family cytokines act directly on basophils to influence their function. For example, IL-18 induces IL-4 and IL-13 production by basophils when combined with a second stimulus, particularly IL-3, and this accounts in part for the ability of IL-18 to promote Th2-type immune responses. IL-33 synergizes potently with IL-3 to augment IL-4, IL-5, IL-6 and IL-13 production by basophils (Smithgall MD *et al.*, 2008, Theoharides TC *et al.*, 2010).

IL-18 has a crucial role in priming Natural killer (NK) cells for subsequent IFN- γ production and augments its release in response to IL-12. IL-18 also enhances the cytolytic functions of NK cells by inducing increased expression of perforin and FAS ligand. IL-33, despite being associated with Th2-type responses, induces IFN- γ production by NK cells after induction of its receptor by IL-12. IL-18 and IL-33 also influence NKT cell function. Both cytokines synergize with IL-12 to drive antigen-

independent expression of IFN- γ by NKT cells, and enhance their production of IL-4, IL-5, IL-13, GM-CSF and TNF- α following T cell receptor (TCR) stimulation. Thus, the actions of IL-18 and IL-33 on NK and NKT cells probably contribute to early immune responses against microbial or viral infection. Many, but not all, DC subsets produce IL-1 and IL-18. They also respond to IL-1 and IL-18, often in combination with other cytokines, by upregulating the expression of CD40, OX40L and IL-12. These responses to IL-1 and IL-18 increase the T cell stimulatory ability of DCs.

Although little is known about the effect of IL-1F6, IL-1F8 and IL-1F9 on cells of the innate immune system, the receptor of these cytokines, IL-1RL2, is expressed by macrophages and DCs, so it is likely that they affect the biology of these cells. IL-1F7 tempers the macrophage response to various activating stimuli.

Effects on adaptive immune cells

By their activating and cytokine-inducing effects on cells of the innate immune system, IL-1 family members also serve as a crucial link in translating innate immune responses into the appropriate adaptive immune response.

IL-1 has been known for a long time to promote T cell responses, but recent findings have identified a key role for IL-1 in the differentiation of Th17 cells as well as in the promotion of IL-17- and IL-22-producing memory T cell recall responses in both mice and humans, has been suggested. In addition, antigen-presenting cell-derived IL-1 acts directly on both naive and memory T cells to enhance their expansion and survival. IL-1 enables T cell proliferation by upregulating IL-2R expression and activates several signalling pathways that have anti-apoptotic effects (such as the NF- κ B pathway). The increase in T cell numbers in the presence of IL-1 results in enhanced and prolonged primary and secondary T cell responses, especially of Th2 and Th17 cells, and in enhanced help for antibody production by B cells. In particular, IL-33 amplifies cytokine

production by committed Th2 cells, whereas IL-1 promotes their proliferation, although it has little effect on cytokine secretion. IL-18 is typically described as a Th1-promoting cytokine, and IL-18R is uniquely expressed by Th1 cells in response to IL-12 stimulation. IL-18 is dispensable for the initial polarization of Th1 cells, but provides an accelerating and amplifying signal for their proliferation and IFN- γ production. However, in the presence of the correct co-stimuli, IL-18 can also induce the production of Th2-type cytokines by both naive and Th1-polarized cells. So, in addition to the specific effects described below, IL-1 is a general amplifier of T cell responses.

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.

Excessive production of IL-18 and IL-33 is found in both blood and inflamed joints of patients with rheumatoid arthritis (Kunisch *et al.*, 2012) 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. 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 . 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. Both IL-1 α and IL-1 β 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. 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.

The expression of IL-1 β 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-1 α and IL-1 β 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 hyper-responsiveness (AHR). IL-1Ra administered at the allergen challenge phase reduces inflammation and AHR in response to ovalbumin. 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 and skin diseases

IL-1 family members are abundantly expressed in the skin. Langerhans cells produce IL-1 β and IL-18 following activation by cytokines, microbial products or allergens. IL-1 β 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. Langerhans cell-derived IL-1 β and IL-18 also induce keratinocytes to make neutrophil-attracting chemokines and inflammatory cytokines, thus initiating a complete immune response to pathogens. Both IL-1 α and IL-1 β are required for contact hypersensitivity reactions, although different reports have implicated one or the other. IL-18 also promotes contact hypersensitivity, and inhibition of IL-18 in rodents alleviates ongoing dermatitis. In our recent study, we found that, as IL-1 α , IL-1 β and IL-18, even the other members of IL-1 family seem to be involved in ACD pathogenesis. IL-

IL-1F3 and IL-1F6, IL-1F8 and IL-1F9 expression was enhanced in lesional skin of ACD patients, but no increase was detected in non lesional skin; a likely scenario was observed *ex vivo*, where after haptens challenge, a significant difference was detected between uninvolved skin of ACD patients and healthy skin. Conversely, IL-1F5 was not augmented *in vivo*, as well as *ex vivo*. Thus, the absence of a negative regulation represented by IL-1F5, seems to be responsible for the increased proinflammatory activity of IL-1F6, IL-1F8 and IL-1F9. IL-1F5 may represent a promising candidate as a novel anti-inflammatory therapeutic. However our knowledge of the functions of IL-1 family members remains limited, and further studies of the biological mechanisms and disease associations are warranted. Atopic dermatitis is usually thought to be a TH2 cell-driven disease. Given the contributions of IL-33 towards TH2-type responses and the fact that IL-33 is highly expressed in skin, it is possible that IL-33 has a significant role in this disease. Indeed, a polymorphism that leads to increased transcription of *IL1RL1* is overrepresented in patients with atopic dermatitis, but this connection has not been further studied. Serum IL-18 concentrations are increased in patients with atopic dermatitis, and common allergic sensitizers, such as cedar pollen, increase IL-18 expression in the skin. Inhibition or deletion of IL-18 protects against the development of dermatitis in a clinically relevant model of atopic dermatitis and transgenic overexpression of IL-18 in the skin leads to high levels of IgE, a hallmark of this disease¹²⁴. These effects are probably due to the ability of IL-18 to promote TH2-type cytokine production and enhance Langerhans cell function. Transgenic overexpression of caspase 1 in the skin of mice also leads to the production of high levels of IgE, as well as TH2-type cytokines and histamine, which contributes to atopic dermatitis-like lesions and excessive scratching. Genetic deletion of IL-18 prevents the development of dermatitis in these mice, and loss of IL-1 delays the progression, suggesting IL-18 and IL-1 have an initiating and accelerating role, respectively.

Recent studies showed that IL-1 family contributes to psoriasis pathogenesis (Cowen EW *et al.*, 2012, Blumberg H *et al.*, 2007, Balato A, *et al.*, 2013, Carrier Y *et al.*, 2011, Johnston A *et al.*, 2011, Muhr P *et al.*, 2011). We have previously showed that IL-33 may be involved in psoriasis biology via keratinocytes (KCs) and mast cells (MCs). Carrier *et al.* reported that IL-36 were effector cytokines downstream T helper (Th)17 cascade. Johnston *et al.* have demonstrated that these cytokines drive skin antimicrobial peptides (AMPs) expression, especially b-defensin-4 (HBD2). Muhr *et al.* have showed a dominance of IL-1 agonist members in psoriatic KCs. Moreover, Cowen *et al.* has reported familial and sporadic cases of generalized pustular psoriasis caused by deficiency of the IL-36Ra (DITRA). In addition, VitD has been shown to suppress TNF- α -induced IL-1a in KCs (Kong J *et al.*, 2006).

Chapter III

Experimental Design

Objective:

Given the recognized involvement of IL-1 family in psoriasis, the immunosuppressive activity of vitD and RA and their therapeutic role in psoriasis, in the present study we investigated the effects of vitD and RA on the expression of IL-1 family cytokines.

Materials and methods

The experimental protocol was approved by the Ethics Committee of the University of Naples Federico II and conformed to the principles outlined in the Declaration of Helsinki. A written informed consent was given by every subject involved in the study.

Expression of IL-1 family members in psoriasis

1 lesional skin biopsy (3 mm diameter) was performed to each of 10 different psoriatic patients for mRNA examination through quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). No systemic treatment for psoriasis was administered for at least one month before the onset of the study. Normal skin derived from 10 healthy donors undergoing to plastic surgery was used as control.

Stimulation assays in healthy skin organ cultures

Skin biopsies (1cm X 1cm) taken from 10 healthy subjects were cultured at 37°C, 5% CO₂ in a 12- well culture plate with a transwell filter (pore size 1 µm; Beckton Dickinson Labware, Franklin Lakes, NJ) containing 1 ml medium (Dulbecco's modified Eagle's medium DMEM, 10% fetal bovine serum FBS, 2 mM L-glutamine and 100 IU/ml penicillin G, 100 µg/ml streptomycin; GIBCO Grand Island, NY) with the epidermis facing upwards at the liquid-air interface and the dermis suspended in the medium.

TNF- α (20 ng/ml GIBCO), IL-17 (10 ng/ml Biosource International, Camarillo, CA), vitD (1,25D3, 10⁻⁸M, Sigma-Aldrich, St Louis, MO), RA (all-trans-RA, 10⁻⁶ M, Sigma-Aldrich) and broadband ultraviolet B (UVB) (100mJ/cm² Philips TL-12, Philips Electronics, Koninklijke, Netherlands) were incubated for 24h when RNA was extracted.

Stimulation assays in normal human primary keratinocytes (KCs)

Primary KCs were isolated from healthy donors undergoing plastic surgery, as previously described [1]. Cell viability was determined using trypan blue coloration. Dose dependent responses on IL-1 β were performed as follows: RA (10⁻⁵ and 10⁻⁶ and 10⁻⁷ M) was dissolved in dimethylsulfoxide (DMSO 0.1% in medium) whereas vitD (10⁻⁷, 10⁻⁸ and 10⁻⁹ M) in ethanol (ETOH 0.001% in medium) and gene expression was assessed at 24h. Stimulation assays were performed as follows: IL-17 (10 ng/ml) and vitD (10⁻⁸M) were incubated for 24h when RNA was extracted.

Stimulation assays in lesional psoriatic skin organ cultures

3 lesional skin biopsies (6 mm diameter) were performed to each of 10 different psoriatic patients; lesional psoriatic skin biopsies were cultured as previously illustrated for healthy skin organ cultures and incubated for 24h with vitD (10^{-8} M) or RA (10^{-6} M) when stored in OCT for immunohistochemical analysis.

RNA extraction, cDNA synthesis and RT-PCR

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) was used to analyze IL-1 β , IL-1Ra, IL-36 α , IL-36 β , IL-36 γ , IL-36Ra, IL-33 and HBD2 gene expression according to the manufacturer's instructions. Values were normalized to the housekeeping gene 18S.

Immunohistochemistry

5 μ m sections were fixed with cold methanol and vectastain Elite ABC Kit (Vector laboratories, Burlingame, CA) was used. Primary monoclonal antibody IL-33 (1/200, clone Nussy-1, Alexis Biochemicals, San Diego, CA) was incubated overnight at 4°C. The sections were then incubated with biotinylated secondary antibody for 30 min at room temperature. Peroxydase activity was revealed using DAB substrate (ImmPACT DAB, Burlingame, CA). Counterstaining was executed with hematoxylin.

Statistical analyses

All 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

To better define the role of IL-1 family in psoriasis, we assessed the expression of IL-1 β , IL-1Ra, IL-36 α , IL-36 β , IL-36 γ , IL-36Ra and IL-33 in plaque psoriatic skin. IL-1 family members gene expression was significantly increased in psoriatic lesions compared with healthy controls. The increase of agonist members was more evident respect to antagonists (Fig. 3a-b).

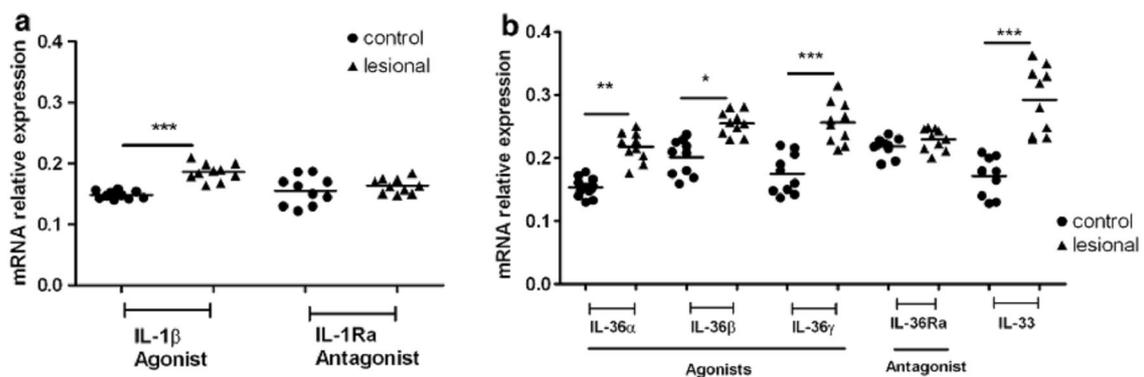


Fig.3 IL-1 family members gene expression in psoriatic (lesional) and healthy (control) skin. Values are expressed as mRNA relative to the housekeeping gene 18S. Data are displayed as dot plot, the line represents the mean. a IL-1 β (agonist) versus IL-1Ra (antagonist).

To investigate the role of IL-1 family members in skin inflammation, we assessed how their gene expression was modulated by inflammatory *stimuli* such as TNF- α , IL-17, and UVB. IL-17 and TNF- α increased all IL-1 family members in *ex vivo* healthy skin organ culture, except for IL-1Ra, being IL-17 a significant more potent inducer of all cytokines (Fig. 3c-h), confirming the axis TNF- α , IL-17 and IL-1 family in the skin. Similarly, TNF- α and IL-17 induced HBD2 too; this AMP was investigated as a positive control. (Fig. 3). Moreover, agonist members (IL-36 α , IL-36 β , IL-36 γ) and IL-33 mRNA were all significantly increased compared with not stimulated skin after UVB irradiation; conversely, antagonist members (IL-1Ra, IL-36Ra) and HBD2 were not statistically increased (Fig. 3c-h). These data showed that UVB-induced pro-inflammatory pathway

caused a major increase of agonists respect to antagonists; HBD2 confirmed the validity of experimental setup, as previously reported. Next, we analyzed the effects of vitD and RA. First of all, we investigated their effects on normal primary KCs viability; no significant modifications were assessed by the solvents as well as increasing doses of vitD and RA (Fig.4).

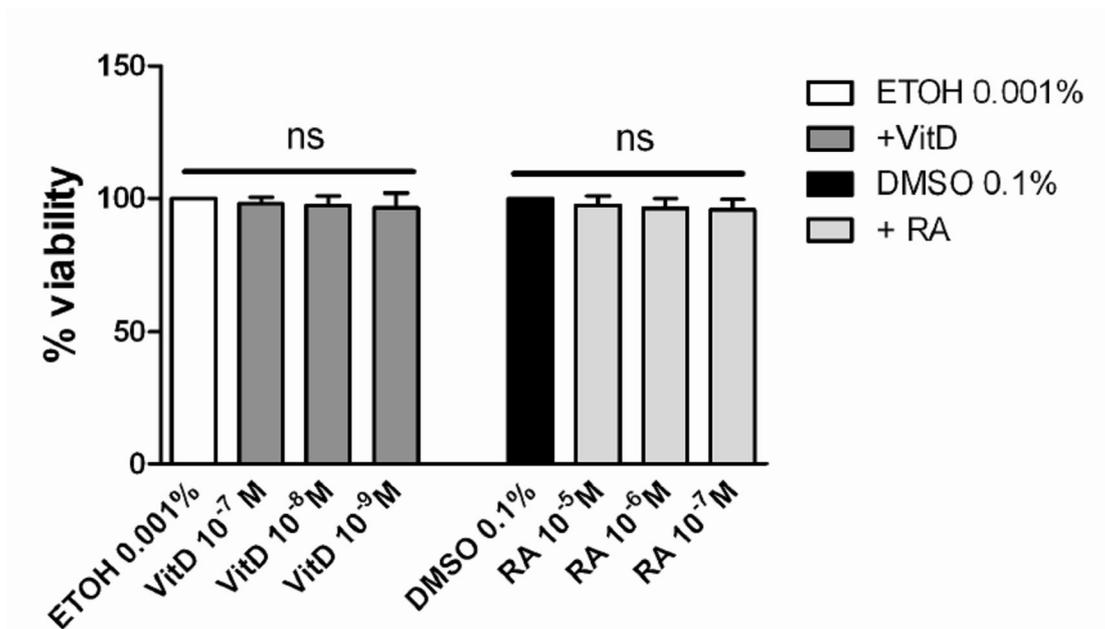


Fig.4 Primary keratinocytes viability after vitD and RA incubation for 24h. Cell viability was investigated following different concentrations of vitD (1,25D3, 10⁻⁷,10⁻⁸ and 10⁻⁹ M) dissolved in ethanol (ETOH 0.001% in medium) or RA (all-*trans*-RA, 10⁻⁵, 10⁻⁶ and 10⁻⁷ M) dissolved in dimethylsulfoxide (DMSO 0.1% in medium). For statistical analyses Unpaired Student's *t*-test was performed. Data are expressed as means ± SD of five independent experiments, each performed in duplicate.

Secondly, we investigated dose response effects of vitD and RA on IL-1β expression; no significant alterations were encountered for both (Fig.5) so that 10⁻⁸ M (vitD) and 10⁻⁶ M (RA), physiological values (Buck J *et al.*, 1990, Tremezaygues L *et al.*, 2011) were chosen for next experiments.

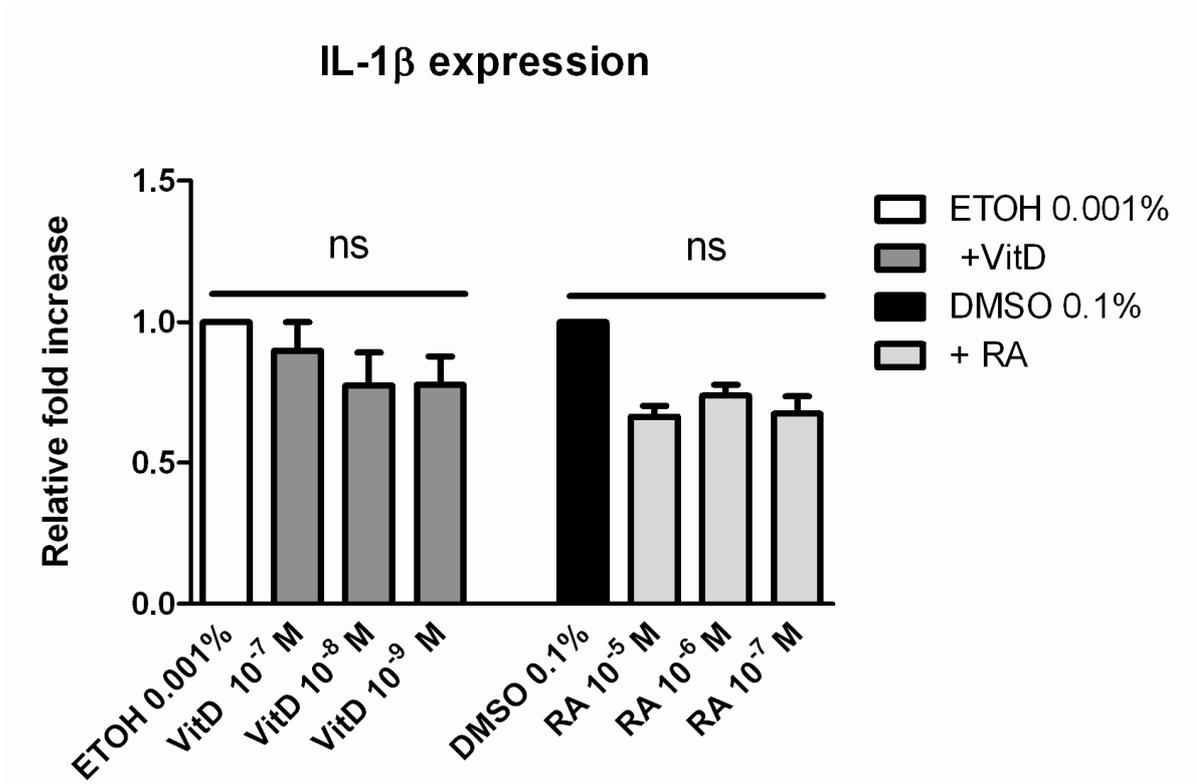


Fig.5 Primary keratinocytes dose dependent responses of vitD and RA on IL-1 β gene expression. Dose dependent responses of primary keratinocytes following different concentrations of vitD (1,25D3, 10⁻⁷, 10⁻⁸ and 10⁻⁹ M), dissolved in ethanol (ETOH 0.001%) or RA (all-*trans*-RA, 10⁻⁵, 10⁻⁶ and 10⁻⁷ M), dissolved in dimethylsulfoxide (DMSO 0,1%). For statistical analyses Unpaired Student's *t*-test was performed. Data are expressed as means \pm SD of five independent experiments, each performed in duplicate.

VitD and RA were able to induce a not significant increase for almost all analyzed cytokines, whereas IL-36 β and HBD2 were up-regulated by vitD (Fig. 6a-g). RA revealed to be an inhibitor stronger than vitD for all cytokines apart from IL-33; a similar scenario was depicted for HBD2 too (Fig. 6g).

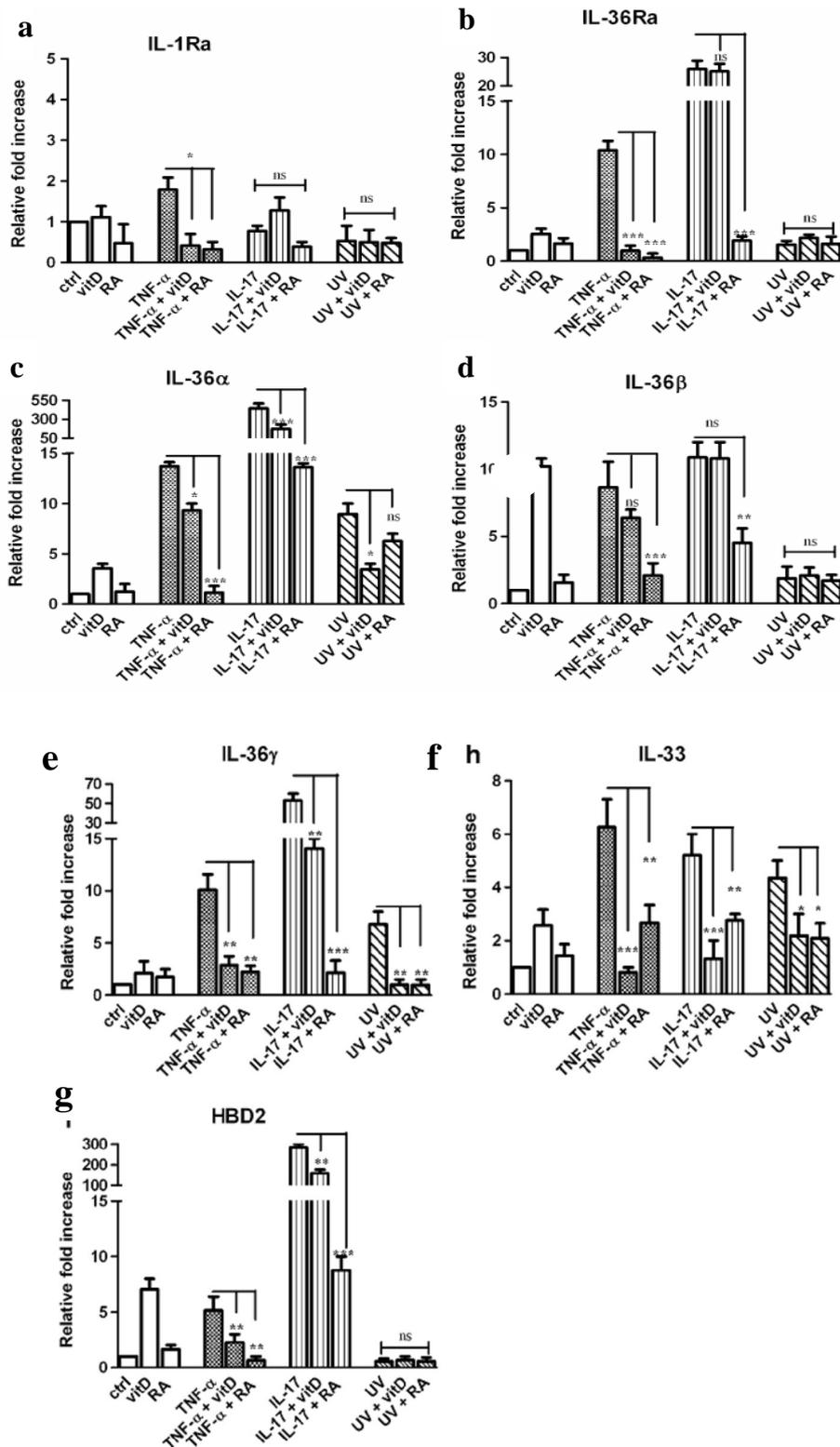


Fig.6 IL-1 family members gene expression in healthy skin organ culture. IL-1 family members gene expression in stimulation assays performed *ex vivo* healthy skin organ cultures. TNF- α , IL-17, UVB, vitD and RA were incubated for 24 h. Values are expressed as relative fold increase, calculated with delta-delta CT method, normalized to the housekeeping gene 18S, and compared with untreated *ex vivo* skin. Data are displayed as mean \pm SD of five independent experiments, each performed in duplicate; a IL-1Ra, b IL-36Ra, c IL-36 α , d IL-36 β , e IL-36 γ , f IL-33, g HBD2. For statistical analyses, unpaired Student's t test was performed. Ns not statistically significant; * P(0.05); ** P(0.01); ***P(0.001)

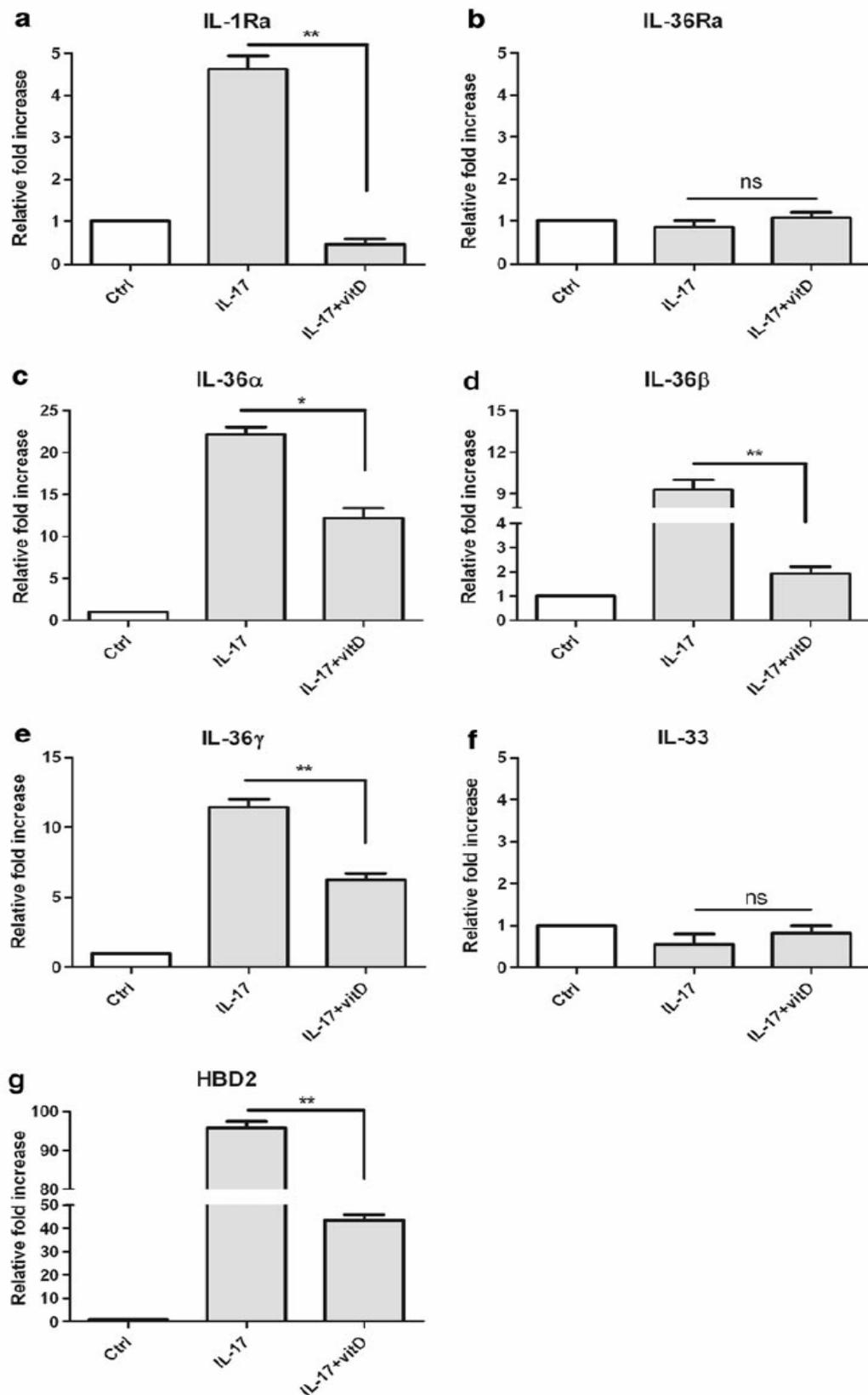


Fig. 7 IL-1 family members gene expression in stimulation assays performed in primary normal human KCs. IL-17 and vitD were incubated for 24 h. Values are expressed as relative fold increase, calculated with delta-delta CT method, normalized to the housekeeping gene 18S, and compared with untreated KCs. Data are displayed as mean \pm SD of five independent experiments, each performed in duplicate; a IL- 1Ra, b IL-36Ra, c IL-36a, d IL-36b, e IL-36c, f IL-33, g HBD2. For statistical analyses, unpaired Student's t test was performed. Ns not statistically significant; *P<0.05; **P<0.01; ***P<0.001

Given the activity of IL-17 to induce HBD2 and the effect of vitD to reduce this, the reported action of IL-36 β as an inducer of HBD2 [17,25], we sought to deeper investigate the relationship between vitD, IL-17, IL-1 family members and HBD2. To clarify whether IL-1 family members were involved in the action of vitD in KCs and if this linkage was promoted by IL-17, as for HBD2, we stimulated KCs with IL-17 and subsequently treated with vitD. Our results showed that vitD suppressed IL-36 α and IL-36 γ as well as HBD2 expression, induced by IL-17 (Fig. 2 a-g). These results were comparable to skin organ culture ones, except for IL-36 β , that was not suppressed by vitD; since vitD alone induced IL-36 β in the skin but not in KCs (data not shown), this data indicates that other cell types may contribute to IL-36 β production. Moreover, IL-36 α and IL-33 were not enhanced by IL-17 in KCs, suggesting that other cells may co-participate in their production (Fig. 2 b,f). VitD was able to downregulate IL-17-induced IL-1Ra in KCs, whereas in skin organ culture IL-17 was not able to enhance it, implying that KCs are involved in this pathway (Fig. 2a).

Since IL-33 gene expression was strongly increased in plaque psoriasis skin, after stimulation with pro-inflammatory *stimuli* and reduced by vitD as well as RA, we sought to analyze the effects of vitD and RA on IL-33 at translational level. Upregulation of IL-33 in the epidermis and in the dermis was evident in psoriasis plaques (Fig. 3a). Significant suppression of IL-33 was observed following treatment with vitD (Fig. 3b) and RA (Fig. 3c).

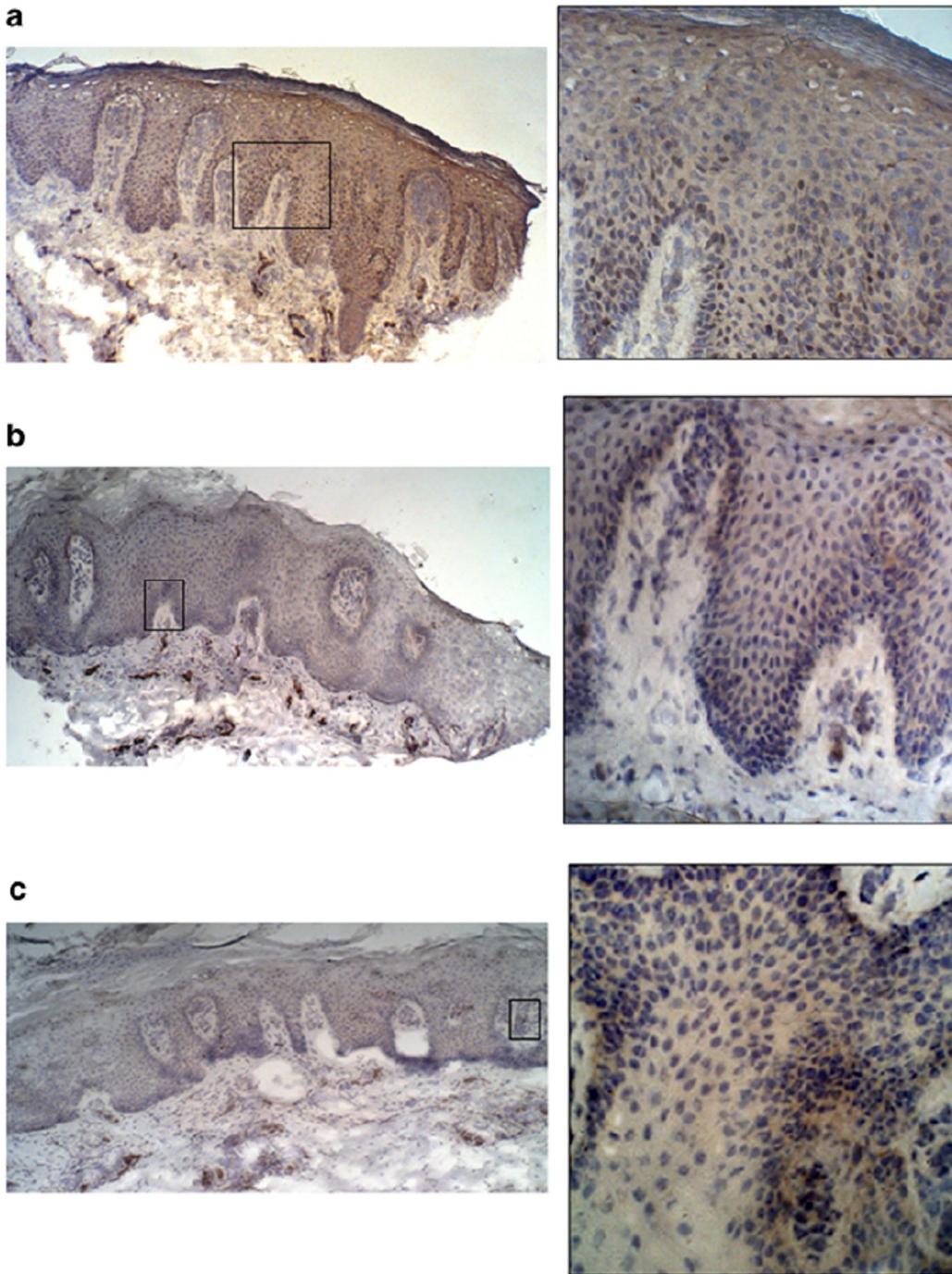


Fig. 8 Immunohistochemical analysis of IL-33 in lesional psoriatic skin before (a) and after treatment with vitD (b) or RA (c). DAB staining x6 (left),x25 (right). Each picture is representative of data from 10 different patients affected by moderate-to-severe psoriasis

Discussion

Our study showed that IL-1 family members were over-expressed in psoriatic plaque skin, being in line with recent studies (Cowen EW *et al.*, 2012, Blumberg H *et al.*, 2007, Balato A, *et al.*, 2013, Carrier Y *et al.*, 2011 Johnston A *et al.*, 2011, Muhr P *et al.*, 2011). We found that the increase of agonist members was more evident respect to antagonists confirming that these novel cytokines may have a pathogenetic role in psoriasis. In *ex vivo* healthy skin organ culture, TNF- α and IL-17 were able to increase almost all IL-1 family members, being IL-17 a significant more potent inducer of all cytokines, confirming the axis TNF- α , IL-17 and IL-1 family in skin inflammation. Similarly, also HBD2 was induced by TNF- α as well as IL-17, in line with Peric *et al.* (Peric M *et al.*, 2009)

The data presented here are in support of recently published findings that showed a similar IL-1 family regulation upon IL-17 stimulation, but in psoriasis-derived KCs (Muhr P *et al.*, 2011). Johnston *et al.* reported that TNF- α , IL-1 α , IL-17 and IL-22 induced IL-36Ra, IL-36 β and IL-36 γ expression by KCs (Johnston A *et al.*, 2011). Debets *et al.* found that, stimulation of KCs with IL-1 β /TNF- α , significantly up-regulated the expression of IL-36 γ and to a lesser extent of IL-36Ra (Debets R *et al.*, 2001) Regarding IL-33, our observations are in line with Muhr *et al.* who reported no variations of IL-33 expression in KCs stimulated with IL-17 (Muhr P *et al.*, 2011). Conversely it was enhanced by TNF- α , IL-17 and UVB in skin organ culture fitting with Byrne *et al.* results (Byrne SN *et al.*, 2011).

Moreover, agonist members (IL-36 α , IL-36 β , IL-36 γ) as well as IL-33 mRNA were all significantly increased after UVB irradiation, whereas antagonists (IL-1Ra, IL-36Ra) were not statistically induced (Fig. 1c-h), indicating that IL-1 family agonists are involved in UVB-induced pro-inflammatory pathway. (Byrne SN *et al.*, 2011). These data

confirmed the involvement of IL-1 family members in response to skin inflammation and extend the notion that these cytokines may contribute to the pathogenesis of psoriasis.

Our findings also showed that enhanced gene expression of IL-1 family members by pro-inflammatory *stimuli* (TNF- α , IL-17, UVB) was suppressed by RA and vitD, being the last one less effective, especially when the induction was operated by IL-17. Furthermore, both RA and vitD were able to suppress IL-33 in psoriatic skin. Because of the increasingly prominent roles of IL-1 family members in psoriasis there is great interest in the relationship between IL-1 family members and RA/vitD (Elias KM *et al.*, 2008). The present data point to a role of RA and vitD as the likely candidates for inhibiting pro-inflammatory effects of these cytokines. Our results are supported by Kong *et al.* who reported that vitD led to reduction of IL-1 α activity and suppressed the biosynthesis of IL-18 (Kong J *et al.*, 2006) and by Ikeda *et al.* who found that vitD as well as RA effectively suppressed mRNA expression of IL-1R1 in human T cells (Ikeda U *et al.*, 2010).

In conclusion, our findings showed an interaction between RA as well as vitD and pro-inflammatory *stimuli* (TNF- α , IL-17, UVB) signalling in the regulation of IL-1 family members. The suppression of IL-1 family members by RA and vitD may have broad implications, considering the wide therapeutic potentials of these 2 molecules and the possible involvement of these cytokines in a relatively large number of autoimmune and inflammatory diseases. To this end, the present study has opened an avenue for more investigations in the future.

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