UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI
MEDICINA

SCUOLA DI DOTTORATO DI
SCIENZE DELLA VITA E DELLA SALUTE

DOTTORATO DI RICERCA IN
SCIENZE MEDICHE CLINICHE E SPERIMENTALI

CICLO XXIV/ANNO 2009

TITOLO DELLA TESI DI DOTTORATO

MECHANISMS OF THE IL-22 ACTIVITY REGULATION IN HUMAN PRIMARY KERATINOCYTES

S.S.D. MED/35

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1. Riassunto

L'Interleuchina (IL)-22, in gran parte prodotta dalle cellule T helper (Th) di tipo Th17 e Th22, è considerata una citochina chiave nella patogenesi della psoriasi poiché, responsabile della marcata iperplasia e dell'alterato differenziamento tipici della cute lesionale. In particolare, l'IL-22 induce la proliferazione dei cheratinociti, inibisce il loro differenziamento terminale e stimola la produzione di peptidi antimicrobici e chemochine. *Signal transducer and activator of transcription* (STAT) 3 è il mediatore principale del segnale indotto dall'IL-22 e la sua completa attivazione richiede la fosforilazione dei residui di tirosina (Tyr) 705 e serina (Ser) 727. La fosforilazione di STAT3 in Tyr705 è inoltre proporzionale all'acetilazione del residuo di lisina (Lys) 685. I livelli di acetilazione di STAT3 sono finemente regolati da processi di acetilazione e deacetilazione controllati, rispettivamente, dagli enzimi acetilasi p300 ed istone deacetilasi (HDAC). Dato che STAT3 appare altamente attivato nell'epidermide psoriasica, lo scopo del lavoro è di identificare molecole target capaci di contrastare l'attivazione di STAT3 mediata dall'IL-22 in cheratinociti umani. Per tale ragione è stato studiato l'effetto di Sirtuina (SIRT) 1, il rappresentante più caratterizzato della famiglia degli enzimi HDAC di classe III, nel controllo dell'attivazione di STAT3 e degli effetti biologici indotti dall'IL-22 in cheratinociti umani. E' stato, infatti, precedentemente dimostrato che SIRT1 è in grado di deacetilare STAT3 nella Lys685 e, di conseguenza, di inibirne la fosforilazione in Tyr705 in cellule epatocitarie. E' stato inoltre riportato che la sirtuina induce il differenziamento e blocca la proliferazione di cheratinociti umani. L'attività di ricerca ha permesso di dimostrare che la proteina SIRT1 è costitutivamente espressa in cellule primarie umane cheratinocitarie, dove essa contribuisce efficientemente alla deacetilazione di STAT3. Durante i processi d'infiammazione cutanea, caratterizzati dalla presenza concomitante dell'Interferone (IFN)-γ e dell'IL-22, l'IFN-γ riduce l'espressione di SIRT1, consentendo così all'IL-22 di attivare STAT3. La riduzione dei livelli di SIRT1 determina, infatti, l'accumulo di STAT3 acetilato in Lys685 e favorisce così la fosforilazione di STAT3 in Tyr705 e le risposte dei cheratinociti all'IL-22. SIRT1 modula negativamente il *Proliferating cellular nuclear antigen* (PCNA), la ciclina D1 ed il *phospho-Retinoblastoma* (pRB), molecole indotte dall'IL-22 attraverso STAT3 e che giocano un ruolo fondamentale nella proliferazione cellulare. Inoltre, la sirtuina aumentando i livelli di cheratina (KRT)1, contrasta gli effetti anti-differenziativi esercitati dall'IL-22 in cheratinociti. Nelle lesioni psoriasiche, l'inibizione di SIRT1 potrebbe essere responsabile della forte attivazione di STAT3 e, quindi, delle risposte esagerate dei cheratinociti all'IL-22 e ad altre citochine pro-infiammatorie che segnalano attraverso STAT3 (IL-6 e oncostatina M). Un'altra molecola potenzialmente capace di controllare le

cascate molecolari e gli effetti biologici indotti dall'IL-22 in cheratinociti umani è il *Suppressor of cytokine signalling* (SOCS) 3. In cellule epatocitarie, infatti, l'aumento dell'espressione di SOCS3 riduce l'attivazione di STAT3 mediata dall'IL-22. Tale studio ha permesso di dimostrare che SOCS3 è l'unico membro della famiglia dei SOCS ad essere indotto dall'IL-22 in cheratinociti umani. Anche se i livelli di SOCS1 non sono modulati dall'IL-22, la sua aumentata espressione in cheratinociti trasfettati stabilmente, inibisce le fosforilazioni di STAT3 in Tyr705 e Ser727 mediate dall'IL-22 in modo simile ai cloni SOCS3. Parallelamente, la trasfezione transiente dei cheratinociti con plasmidi per SOCS1 e SOCS3 riduce l'attività trascrizionale di STAT3 dipendente dall'IL-22. Viceversa, SOCS2 non è coinvolto nella regolazione dell'attivazione di STAT3. Conseguentemente all'inattivazione di STAT3, cheratinociti che esprimono alti livelli di SOCS1 e SOCS3 proliferano di meno dopo trattamento con IL-22 rispetto alle cellule MOCK. Al contrario, l'effetto mitogenico dell'IL-22 è potenziato in cheratinociti che non esprimono il SOCS3. Inoltre, SOCS3 è in grado di ridurre l'espressione di geni pro-infiammatori e di opporsi al de-differenziamento cheratinocitario dipendente dall'IL-22. Gli effetti inibitori di SOCS3 sull'attivazione di STAT3 dipendente dall'IL-22 sono esercitati dalla sua regione KIR (*Kinase Inhibitory Region*). L'uso di attivatori di SIRT1 o di molecole che mimano la regione KIR del SOCS3, in grado quindi di ridurre la fosforilazione di STAT3 in Tyr705 in cheratinociti epidermici, potrebbe essere utile per la cura della psoriasi, ma anche di altre patologie cutanee caratterizzate da un'aberrante attivazione di STAT3.

2. Abstract

Interleukin (IL)-22 is a cytokine mainly released by T helper (Th) 17 and Th22 lymphocytes having a pathogenetic role in psoriasis. In this skin disorder, IL-22 is responsible for the altered proliferative and differentiative processes observed in the epidermis, and induces inflammatory molecules in keratinocytes. Signal transducer and activator of transcription (STAT) 3 is the principal mediator of IL-22 signaling and its complete activation requires the phosphorylation in tyrosine (Tyr) 705 and in serine (Ser) 727 residues. Moreover, STAT3 phosphorylation in Tyr705 is proportional to the acetylation in lysine (Lys) 685 residue. STAT3 acetylation is tightly regulated by the acetylation and deacetylation processes, which are controlled by p300 acetylase and histone deacetylase enzymes (HDAC), respectively. Due to the aberrant STAT3 activation in psoriatic epidermis, the aim of this study was to identify the target molecules able to inhibit the IL-22-triggered STAT3 phosphorylation and down-stream effects in human keratinocytes. For this purpose the role of Sirtuin (SIRT) 1, the most characterized Class III HDAC family member, has been studied in the control of IL-22-dependent signaling in keratinocytes. Indeed, it has previously demonstrated that SIRT1 is able to deacetylate STAT3 in Lys685 residue and, consequently, to inhibit STAT3 phosphorylation in Tyr705 in hepatocyte cells. Moreover, it was reported that the sirtuin is able to induce the differentiation and inhibit the proliferation of human keratinocytes. The research activity allowed us to demonstrate that SIRT1 is constitutively expressed by keratinocytes and that it efficiently contributes to STAT3 deacetylation in these cells. During inflammatory skin processes characterized by a concomitant presence of Interferon (IFN)-γ and IL-22, IFN-γ strongly down-regulates the keratinocyte expression of SIRT1 and IL-22 activates STAT3. In fact, SIRT1 decrement determines an accumulation of STAT3 acetylated in Lys685, thus favoring STAT3 phosphorylation in Tyr705 and keratinocyte responses to IL-22. SIRT1 negatively modulates the Proliferating cellular nuclear antigen (PCNA), cyclin D1 and phospho-Retinoblastoma (pRB), that is STAT3-dependent and IL-22-induced molecules and play a fundamental role in cellular proliferation. In addition, the sirtuin, by enhancing the level of keratin (KRT) 1, counter-acts the IL-22-triggered de-differentiate effect in keratinocytes. In psoriatic lesions, SIRT1 inhibition could be responsible for the strong activation of STAT3 and, thus, for the exaggerated responses of epidermal keratinocytes to IL-22 and other pro-inflammatory cytokines signaling through STAT3 (*i.e.*, IL-6 and oncostatin M). Suppressor of cytokine signalling (SOCS) 3 represents another possible candidate able to control the IL-22-derived molecular cascades and the biological effects in keratinocytes. Indeed, in hepatocyte cells, the enhanced expression of SOCS3 level reduces the IL-22-

dependent STAT3 activation. In this study it has been demonstrated that SOCS3 is the only SOCS family member induced by IL-22 in human keratinocytes. Interestingly, even though SOCS1 was not up-regulated by IL-22, its over-expression in stably trasfected keratinocytes potently inhibited the IL-22-induced Tyr705 and Ser727 phosphorylations of STAT3, likewise to what observed in SOCS3 clones. Consistently, transient transfection of keratinocytes with SOCS1 or SOCS3 plasmids markedly reduced the IL-22-induced STAT3 transcriptional activity. In contrast, stable or transient SOCS2 over-expression in keratinocytes had no effects on STAT3 activation by IL-22. As consequence of STAT3 inactivation, the IL-22-induced proliferation was impaired in SOCS3 and SOCS1 clones compared to mock-trasfected keratinocytes. *Vice versa*, the mitogenic effect exerted by IL-22 on keratinocytes depleted of SOCS3 was more pronounced. Moreover, SOCS3 reduces the expression of IL-22-dependent pro-inflammatory genes and opposes to the de-differentiative effect of the cytokine in keratinocytes. The SOCS3 inhibitory effect on the IL-22-induced STAT3 activation is executed by SOCS3 KIR (kinase inhibitory region) domain. Therefore, the use of SIRT1 activators or molecules able to mimic SOCS3-KIR region, able to reduce the STAT3 Tyr705 phosphorylation in epidermal keratinocytes, could be therapeutically useful for the treatment of psoriasis as well as other skin diseases characterized by an aberrant STAT3 activation.

3. Introduction

3.1 Clinical and histologic features of psoriasis

Psoriasis is a chronic recurrent inflammatory skin disease estimated to affect 2–3% of the general population [1]. Within the spectrum of cutaneous manifestations of psoriasis, different expressions are seen. Individual lesions may vary in size from pinpoint to large plaques, or even erythroderma [2]. More specifically, the clinical spectrum of psoriasis includes the plaque, guttate, small plaque, inverse, erythrodermic, and pustular variants. The most common and well-recognized morphologic presentation of psoriasis is that of the plaque type. The disease usually is characterized by the formation of demarked erythematous plaques with large scaling. The scales are a result of a hyperproliferative epidermis with premature maturation of keratinocytes and incomplete cornification with retention of nuclei in the stratum corneum (parakeratosis). The mitotic rate of the basal keratinocytes is increased as compared with that in normal skin. As a result, the epidermis is thickened (acanthosis), with elongated rete ridges that form fingerlike protrusions into the dermis (Fig. 3.1). The granular layer of the epidermis, the starting site of terminal keratinocyte differentiation, is strongly reduced or missing [2]. The epidermis becomes infiltrated by neutrophils and activated CD8+ T lymphocytes. Within the dermis, an inflammatory infiltrate composed mainly of CD3+ T lymphocytes, dendritic cells (DC), macrophages, mast cells and neutrophils is observed [1]. Elongated and dilated blood vessels in the dermal papillae represent a further histological hallmark of psoriatic skin lesions.

3.2 Pathogenetic mechanisms operating in psoriasis

Even though successful treatment regimens for the therapy of psoriasis have been established for a long time, the primary pathogenetic mechanism and the cell type(s) involved in the onset of the disease are still under debate. Psoriasis is classically responsive to trigger factors that can induce psoriasis *de novo* or exacerbate skin lesions. Trigger factors range from nonspecific stimuli such as skin trauma to more specific triggers such as pathogens (i. e. streptococci) or drugs (i. e. lithium, interferon (IFN)-α) [1]. All these factors generate a pathogenetic cascade culminating in the expansion of lesional and/or circulating T cells in the psoriatic skin. T-lymphocyte infiltrate present in active psoriatic skin establishes a cytokine milieu that dictates specific and pathogenetic gene signatures in resident skin cells [3]. Thus, cytokine-activated keratinocytes overexpress a number of inflammatory mediators that aberrantly amplify and sustain the psoriasiform tissue reactions [4]. Importantly, intrinsic defects and/or alterations of keratinocytes in their immune response to pro-inflammatory cytokines are fundamental for the

induction of psoriatic processes, as demonstrated in genetically-manipulated mouse systems. In particular, transgenic animals that overexpress the transcription factor STAT3 [5] or that lack the inhibitor of NF-κB kinase-2 (IKK-2) [6] in the epidermis developed skin lesions that closely resembled human psoriasis. Similarly, the abrogation of JunB in keratinocytes triggered in mice a skin phenotype with the histological hallmarks of psoriasis, including marked hyperplasia of the epidermis accompanied by a dense dermal infiltrate of inflammatory cells [7]. The hyperplasia observed in these mice could, in part, depend on the overexpression of S100A8 and S100A9, two antimicrobial peptides that have chemotactic activity and a well-known role in keratinocyte maturation and proliferation processes. The development of psoriatic lesions in mice overexpressing STAT3 in the epidermis depends on the presence of activated T cells, whereas the inflammatory responses occurring in the skin of IKK2-transgenic mice are mediated by TNF-α. Therefore, it is clear that an intrinsically dysregulated interrelation between keratinocytes and cells of both the innate and acquired immune response is a key factor in the pathogenesis of psoriasis. Based on the analysis of infiltrating T cell types, their secreted products, and genetic signatures present in lesional skin, psoriasis has been considered for many years as a type-1 tissue reaction with a prominent role for T cell-derived interferon (IFN)-γ [8]. However, other cytokines and T-cell subsets have been identified as fundamental players during inflammatory responses in psoriasis. They include IL-21-relasing T cells, which have been shown to regulate keratinocyte proliferation in psoriatic skin, Th17 cells and the recently identified IL-22-producing type Th22 cells [9-11]. Both IL-17 and IL-22 belong to a class of cytokines with predominant effects on epithelial cells. Keratinocytes, in fact, are strongly influenced by IL-17 and in response to this cytokine up-regulate chemokines and immune-modulatory molecules [12]. A functional role of Th17 cells in psoriasis is suggested by their reduction during successful anti-TNF-α treatment [13]. IL-22 also acts pathogenetically in psoriatic skin, as it regulates proliferative processes and the expression of innate immunity molecules in psoriatic keratinocytes (see below) [14].

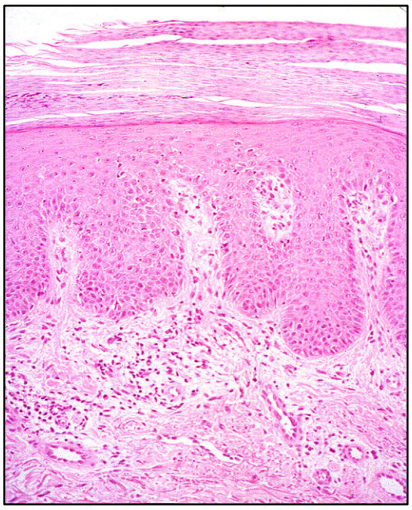

Figure 3.2: Hematoxilyn-eosin staining of a plaque variant of psoriatic skin [2]

3.3 IL-22 and its pathogenetic role in psoriasis

IL-22, together with IL-19, IL-20, IL-24, and IL-26, belongs to a family of cytokines structurally related to IL-10 [15]. IL-22 is mainly produced by mast cells, natural killer (NK) cells and Th22 cells [11, 14]. IL-22 acts through a heterodimeric receptor containing the IL-10 receptor (R) 2 and IL-22R1 chains [16] (Fig. 3.3). IL-10R2 is widely expressed on immune cells, whereas IL-22R1 expression is restricted to non-hematopoietic cells, such as epidermal keratinocytes and epithelial cells of the gastrointestinal tract and the lung [17, 18]. Upon binding to its R1 chain, IL-22 induces a conformational change that enables IL-10R2 to interact with the newly formed ligand-receptor complexes. This, in turn, activates Janus kinase (JAK) 1 and tyrosine kinase (Tyk) 2, leading to the Tyr705 phosphorylation of STAT3 [19]. The tyrosine phosphorylation of STAT3 in the cytoplasm leads to its dimerization, translocation into the nucleus, and DNA binding. Therefore, STAT3 phosphorylation in Ser727 residue is necessary to maximize STAT3 transcriptional activity [20]. IL-22 was also found to activate the Extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p38 Mitogen-activated protein kinase (MAPK) in the rat hepatoma cell line H4IIE [19]. In addition, IL-22 up-regulates the mRNA expression of suppressor of cytokine signalling (SOCS) 3, which abrogates IL-22-induced STAT signaling in hepatic cells through a classical negative feed-back loop [21]. A soluble IL-22-binding protein, IL-22RA2, encoded by a distinct gene has been identified. This soluble receptor, which has 34% amino

acid identity to the extracellular domain of the IL-22RA1, binds IL-22 and antagonizes its functional activities [22] (Fig. 3.3).

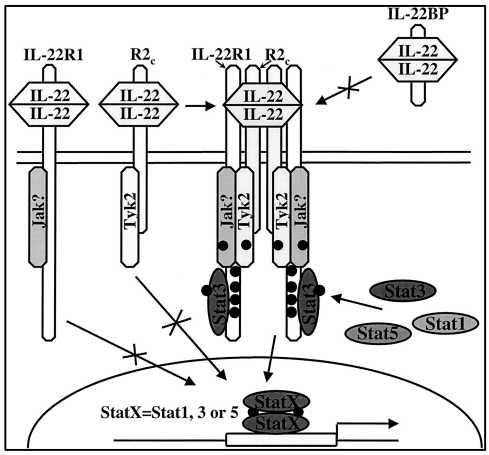

Figure 3.3: Model of the IL-22R complex and signal transduction.

Wolk *et al*. have demonstrated that IL-22 mRNA is massively elevated in psoriatic skin lesions [23]. The increase of the IL-22 mRNA level in lesional skin is much greater than that of the cytokines IFN-γ and IL-1β, at that time viewed as pathogenetically relevant for this disease [18]. Further analyses of samples of psoriasis patients revealed that the local expression of IL-22 is associated with high IL-22 blood plasma levels, whereas again the plasma levels of IFN-γ, IL-17 and IL-1β were only minimally elevated [24]. A correlation analysis demonstrated a clear positive relationship between the IL-22 blood plasma levels and the severity of the disease [24]. After anti-psoriatic therapy a marked reduction of both the cutaneous IL-22 mRNA expression and the IL-22 blood plasma level was seen. In keratinocytes IL-22 regulates a number of genes which can be divided into three functional categories: antimicrobial, differentiation-associated, and mobility regulating genes [24]. The antimicrobial peptides induced by IL-22 in keratinocytes include β-defensin 2 and 3, and the S100 proteins S100A7, S100A8, and S100A9. In addition, IL-22 inhibits the terminal differentiation of keratinocytes, which is a prerequisite for the normal formation of corneocytes of the stratum corneum of the skin and for physiologic desquamation. In particular, the cytokine reduces the expression of genes essential for the keratinocyte differentiation in the stratum spinosum of the epidermis, such as profillagrin, cheratin (KRT) 1, KRT10, desmoccollin-1 and kallikrein-7 [24]. IL-22 promotes the production of cellular mobility associated molecules matrix metalloproteinases (MMP) 1 and 3 and inhibits annexin A9 production. IL-22 induces the production of

chemokines recruiting neutrophilic granulocytes as a prerequisite for migration of these cells into the skin. Moreover, in human skin equivalents and human keratinocyte cultures, IL-22 enhances IL-20 mRNA expression, protein expression, and secretion [25]. IL-20, in turn, amplifies and/or prolongs the IL-22 inhibitory action on keratinocyte differentiation that leads to the characteristic epidermal changes observed in psoriatic epidermis, such as the decrease in the granular cell layer (hypogranulosis) and an increase in epidermis thickness (acanthosis) [26, 27]. Interestingly, the IL-22-induced inhibition of the terminal keratinocyte differentiation does not seem to be associated with an expression change of anti- or pro-apoptotic proteins. However, it coincided with the induction of STAT3 expression that was necessary for this IL-22 effect [26]. These facts, together with the high IL-22 expression levels in psoriatic plaques and in the blood of psoriasis patients, suggest that IL-22 plays a major role in the keratinocyte alterations in psoriasis [18, 24]. This postulated role of IL-22 in the pathogenesis of psoriasis was further supported by three studies in mice: Zheng et al. found that repeated cutaneous application of IL-23 induced acanthosis in mice in an IL-22-dependent way [28]. The Fouser group investigated a psoriasis-like disease model in mice, which is induced by the transfer of T cells to pathogen-free scid/scid mice [29]. In this model, neutralization of IL-22 reduced acanthosis, inflammatory infiltrates, and expression of some cytokines. In the third study, Wolk et al. demonstrated that transgenic overexpression of IL-22 in mice resulted in neonatal mortality and psoriasis-like skin alterations including epidermal acanthosis and hypogranularity [30]. In addition, STAT3 is activated in lesional keratinocytes from psoriatic patients [31]. This up-regulation of STAT3 activation in psoriasis does not appear to be a secondary outcome of epidermal hyperplasia, because lesions from non-psoriatic inflammatory skin diseases with characteristic acanthosis show a STAT3 staining pattern similar to normal epidermis. Moreover, K5.Stat3C transgenic mice, in which STAT3 is constitutively active in keratinocytes, develop psoriasiform lesions after wounding stimuli or topical treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), which strongly suggests that STAT3 activation is required for the development of psoriasis [5]. Expectedly, a topical pre-treatment of K5.Stat3C mice with STAT3 decoy oligonucleotides abrogates the de novo generation of tape stripping induced psoriatic lesions with less T cell infiltrates [32]. In addition, Miyoshi et al. have recently reported that topical treatment with STA-21, an antibiotic which blocks STAT3 dimerization and DNA binding, not only inhibits the development of psoriasiform lesions in K5.Stat3C mice, but also ameliorates psoriatic lesions in six of the eight psoriasis patients, suggesting that an inhibition of STAT3 activation could be a reliable therapy for psoriasis [33].

3.4 Modulation of STAT3 activation

3.4.1 Role of SIRT1

Post-translational modifications of many if not all proteins critically regulate their biological functions. It is now well accepted that these modifications are required to face challenges and stress from the environment, to trigger a variety of processes ranging from cell proliferation, differentiation, and autophagy to apoptosis [34]. These modifications, when not properly regulated, also contribute to multiple pathologies such as cancer and auto-immune diseases. Therefore, several disease-related research programs are currently ongoing in order to better understand the pathways that involve these post-translational modifications and to subsequently define new therapeutic targets. Protein phosphorylation is the most widely studied modification but early evidence suggested that many other protein modifications such as methylation, ubiquitination, sumoylation and lysine acetylation also occur in vivo [35]. Lysine acetylation, defined as the addition of an acetyl moiety to the ε-amino group of a lysine residue, has been linked for many years to gene transcription [34]. Interestingly, it has been reported that STAT3 acetylation in Lys685 residue is essential for the phosphorylation of STAT3 in Tyr705 in hepatic cells [35]. Acetylation of STAT3 relies on a tight balance between acetylation and deacetylation performed primarily by p300 acetylase and histone deacetylase enzymes (HDAC), respectively [35]. There are two protein families with HDAC activity: Class III NAD-dependent HDAC (sirtuins) and the classical HDAC family (Classes I and II). Class I and II use a zinc as cofactor and are inhibited by Tricostatin A (TSA), whereas sirtuins aren't sensible to TSA and use NAD^+ to convert acetylated protein substrates into deacetylated protein, nicotinamide and the acetyl ester metabolites (Fig. 3.4.1) [36, 37]. The human sirtuin family is made up of seven members, SIRT1-7, with each having distinct cellular targets and cellular localizations (Table 3.4.1). Most sirtuins (SIRT1, -3 and -5) catalyze reactions of deacetylation, whereas SIRT4 and SIRT6 are mono-ADP-ribosyl transferases with no deacetylase activity. Acetylated histones H1, H3, and H4 are known to be physiological substrates for the sirtuins, and lysine 16 in histone H4 appears to be the most critical residue for sirtuin mediated transcriptional silencing. However, SIRT1-3 also target non-histone proteins, including various transcription factors [38]. SIRT1 is the best characterized sirtuin member and it is localized to either the nucleus or cytoplasm depending on tissue and cell type [38].

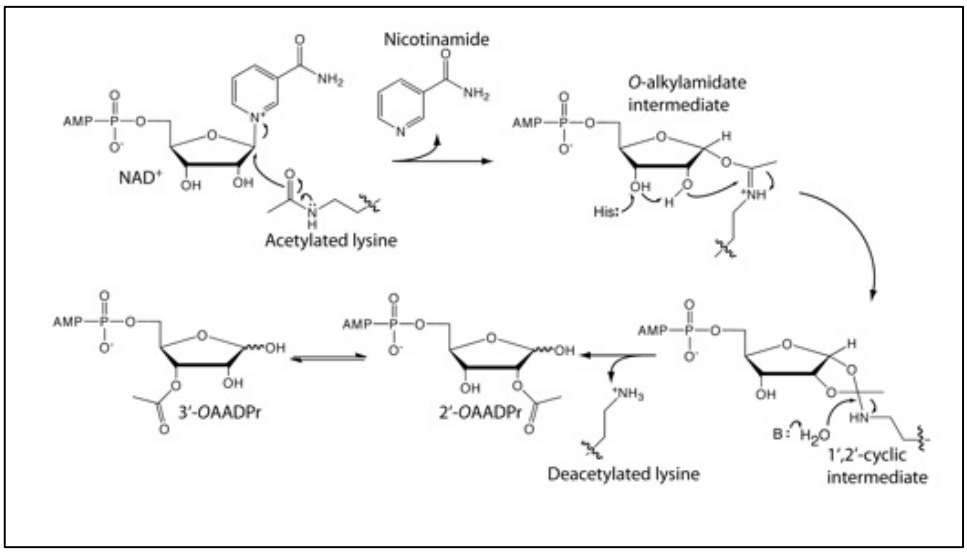

Figure 3.4.1: Reaction of deacetylation catalysed by sirtuins.

It deacetylates several transcription factors involved in the regulation of cellular proliferation, apoptosis, differentiation, metabolism and inflammation [38]. Of note, SIRT1 counteracts STAT3 activation in different cell types [39-41]. In particular, SIRT1-mediated deacetylation of STAT3 in murine skeletal muscle cells determines the reduction of STAT3 binding to the p55α/p50α regulatory subunits of phosphoinositide 3-kinase (PI3K), thereby promoting PI3K signaling activation during insulin stimulation [40]. Moreover, the sirtuin decreases mitochondrial biogenesis and cellular respiration in murine embryonic fibroblasts, through the inhibition of STAT3 expression and serine phosphorylation [41]. Interestingly, SIRT1 plays an important role during keratinocyte proliferation and differentiation. In fact, it has been reported that SIRT1 induces the differentiative programs and inhibits the proliferative processes in human keratinocytes through the regulation of E2F1 transcriptional activity [42].

Sirtuin	Type of enzyme	Subcellular localization
SIRT1	Deacetylase	Cytoplasm and nucleus
SIRT2	Deacetylase	Cytoplasm and nucleus
SIRT3	Deacetylase and ADP-rybosiltransferase	Mitochondria
SIRT4	ADP-rybosiltransferase	Mitochondria
SIRT5	Deacetylase	Mitochondria
SIRT6	Deacetylase and ADP-rybosiltransferase	Nucleus
SIRT7	Deacetylase	Nucleus

Table 3.4.1: Sirtuin function and localization.

These SIRT1-mediated effects are specific of keratinocytes and are not shared by other cell types, such as white adipocytes and myocytes, where SIRT1 overexpression counteracts differentiative processes [43, 44].

3.4.2 Role of SOCS proteins

SOCS proteins and cytokine-inducible SRC homology 2 (SH2)-domain-containing proteins (CIS) comprise a family of intracellular proteins, several of which have been shown to regulate the responses of immune cells to cytokines [45]. There are eight members of the CIS–SOCS family (CIS and SOCS1-7), each of which has a central SH2 domain, an amino-terminal domain of variable length and divergent sequence, and a carboxy-terminal 40-amino-acid module that is known as the SOCS box (Fig. 3.4.2.A). The SOCS box interacts with elongin B and elongin C, cullin-5 and RING-box-2 (RBX2), which recruits E2 ubiquitin transferase [45]. CIS–SOCS-family proteins, as well as other SOCS-box-containing molecules, probably function as E3 ubiquitin ligases and mediate the degradation of proteins that are associated with these family members through their N-terminal regions. In addition to their ability to suppress signaling by ubiquitin-mediated degradation of the signaling complex, both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity directly through their kinase inhibitory region (KIR), which is proposed to function as a pseudosubstrate and is important for the suppression of cytokine signals (Fig. 3.4.2.A) [45].

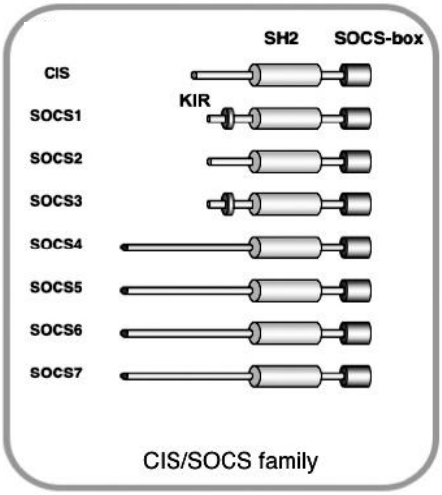

Figure 3.4.2.A: Schematic structure of the SOCS proteins.

In general, the constitutive level of SOCS protein expression in cells is low, but SOCS protein expression is highly inducible, often in a transient manner, upon stimulation with cytokines both *in vitro* and *in vivo* (Fig. 3.4.2B, left scheme). SOCS-mediated down-regulation of cytokine-induced JAK-STAT signaling involves different mechanisms (Fig. 3.4.2B). Via its SH2 domain, SOCS1 binds directly to the JAK and inhibits kinase activity [46]. SOCS3 also inhibits JAK activity, but in contrast to SOCS1, this requires binding between the SH2 domain of SOCS3 and the phosphorylated receptor [47]. Finally, SOCS proteins can inhibit signaling by coupling of signaling proteins to degradation via the proteasomal machinery (Fig. 3.4.2B, right scheme) [45].

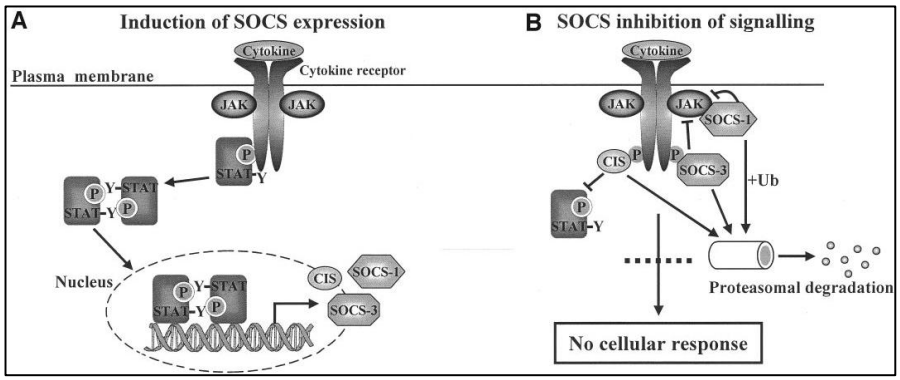

Figure 3.4.2B: SOCS proteins inhibit cytokine signaling.

Induction of SOCS expression by various cytokines is STAT-dependent, and indeed, the promoter regions of the CIS [48], SOCS1, and SOCS3 genes [49] show functional STAT-binding elements. Cytokine-induced expression of SOCS1 and SOCS3 can be inhibited by STAT3 dominant negative mutants [49], while gene expression of CIS can be inhibited by STAT5 dominant negative mutants [50]. From evidence suggesting that forced expression of SOCS3 can inhibit IL-6-mediated STAT3 activation it was proposed that SOCS3, which is induced by STAT3 activation, can act as a negative-feedback regulator of STAT3. For instance, *Socs3* gene therapy, using adenoviral-mediated gene delivery, suppresses the IL-6-induced and STAT3-mediated proliferation of cultured synovial fibroblasts as well as significantly reduced the onset and progression of experimental antigen- and collagen-induced arthritis in mice [51]. Moreover, the reduction of SOCS3 expression and the consequent increase of STAT3 activation contribute to the development of cancer in multiple neoplasias, including cholangiocarcinoma, hepatocellular carcinomas and breast and lung cancer [52]. In addition, cytokine- and growth factor-induced STAT3 activation is inhibited by SOCS3 in keratinocyte cells. In particular, the over-expression of SOCS3 in keratinocytes abrogates the Hepatocyte growth factor (HGF)-derived STAT3 activation and proliferative effects [53]. Furthermore, the keratinocyte-specific conditional ablation of SOCS3 in transgenic mice resulted in an aberrant STAT3 activation associated with keratinocyte hyperproliferation [54]. The role of SOCS3 molecules in the control of IL-22-mediated STAT3 activation in keratinocytes is yet unknown. Of note, IL-22 is able to upregulates the mRNA expression of SOCS3 in hepatic cells [55]. Moreover, SOCS3 overexpression abrogates IL-22-induced STAT3 activation preventing IL-22-induced liver cell regeneration.

4. Materials and Methods

4.1 Tissue samples

For immunohistochemistry, 4-mm punch biopsies were taken from lesional (LS) skin of adult patients with chronic plaque psoriasis and from normal skin of healthy subjects undergoing plastic surgery. For patients with psoriasis, a skin biopsy from non lesional (NLS) skin distant from the plaques was also taken. Keratinocyte cultures were established from 6-mm biopsies obtained from both involved and uninvolved skin of patients with psoriasis and from healthy control subjects. The study was approved by the Ethical Committee of the IDI-IRCCS (Rome, Italy).

4.2 Keratinocyte cultures and treatments

Primary cultures of human keratinocytes were obtained from skin biopsies, as previously described [56]. Keratinocyte cultures were grown in serum-free keratinocyte growth medium (KGM; Clonetics, Walkersville, MD, USA), for 3-5 days (at 60–80% confluence) before performing experiments. Stimulations with 50 ng/ml IL-22, 200 U/ml IFN-γ, 50 ng/ml TNF-α, or 50 ng/ml IL-17 (all from R&D Systems, Minneapolis, MN, USA) were performed in keratinocyte basal medium (KBM, Clonetics). Subconfluent cultures were also stimulated with supernatants from Th1 clones or RPMI medium diluted 1:3 in KBM. Terminal differentiation of keratinocyte cultures was achieved by growing cells at 100% of confluence (*t*0) and, thus, keeping them in culture for another 4 days. Keratinocytes undergoing differentiation were also cultured in the presence of IL-22 and/or IFN-γ. The HaCaT human keratinocyte cell line was a gift from N. E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and was grown in Dulbecco modified Eagle medium (DMEM; Biochrom, Cambridge, UK) supplemented with 10% Fetalclone II serum (HyClone Laboratories, South Logan, UT, USA). CD4+ T-cell clones were obtained from peripheral blood of patients with psoriasis, as previously described [57]. Cells were periodically stimulated with 1% phytohemagglutinin (Invitrogen, Carlsbad, CA, USA), and were activated with plate-coated anti-CD3 and soluble anti-CD28 (both at 1 μg/ml). Clone supernatants were assayed for IFN-γ, TNF-α, IL-17, IL-22, and IL-4 content with a commercially available ELISA kit (R&D Systems). Clones were classified depending on their cytokine profile, and supernatants of Th1 clones containing levels of IFN-γ > 20 ng/ml were used on keratinocyte cultures.

4.3 RNA isolation, polymerase chain reaction (PCR) and real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). mRNA was reverse-transcribed into cDNA and analyzed by real-time PCR or PCR. The mRNA expression of all analyzed genes through real-time PCR procedure was evaluated in the ABI Prism SDS 7000 PCR instrument (Applied Biosystems, Branchburg, NJ, USA), using SYBR Green PCR reagents or Taqman PCR Master Mix. The forward and reverse primers employed were as follows: for CXCL8, 5'-GCTGGCTTATCTTCACCATCATG-3 'and 5'-TTATTTTTTTTCAGTTAATTAACAGATGCTATCAT-3'; for SOCS3, 5'-AAGGACGGAGACTTCGATTCG-3' and 5'-AAACTTGCTGTGGGTGACCAT-3'; for SOCS1, 5'-TTTTTCGCCCTTAGCGTGA-3' and 5'-AGCAGCTCGAAGAGGCAGTC-3'; for STAT3, 5'-GGCGTCACTTTCACTTGGGT-3' and 5'-CCACGGACTGGATCTGGGT-3; for STAT1, 5'-TTGCTTGGATCAGCTGCAGA-3' and 5'-GCTGCAGACTCTCCGCAACTA-3'; for SIRT1, 5'-GCTGGCCTAATAGAGTGGCAA-3' and 5'-CTCAGCGCCATGGAAAATG-3', for β-actin, 5'-CATCGAGCACGGCATCGTCA-3' and 5'-TAGCACAGCCTGGATAGCAAC-3'. The sequences of the primers and internal probe for HBD-2 mRNA have been previously described [58]. Primers for CXCL1 (Hs00236937), S100A7 (Hs00161488), CIS (Hs003203371), SOCS2 (Hs00919620), SOCS4 (Hs00328404), SOCS5 (Hs00367107), SOCS6 (Hs00377781), SOCS7 (Hs00389987) and HPRT1 (Hs01003267) were provided by Applied Biosystems. The levels of gene expression were determined by normalizing to β-actin or HPRT1 mRNA expression. The values obtained from triplicate experiments were averaged, and data are presented as means ± sd. The mRNA expression of sirtuin members was analyzed by Semi-Quantitative PCR conducted in a Thermal Cycler (Applied Biosystems). The primer pairs' specific for the family sirtuin members were as follows: SIRT1 (GenBank NM_012238; oligonucleotide forward 405-428 bp, reverse 563-586 bp); SIRT2 (GenBank NM_012237; oligonucleotide forward 438–461 bp, reverse 699–722 bp); SIRT3 (GenBank NM_012239; oligonucleotide forward 290–313 bp, reverse 527–550 bp); SIRT4 (GenBank NM_012240; oligonucleotide forward 414–437 bp, reverse 565–588 bp); SIRT5 (GenBank NM_012241; oligonucleotide forward 365–388 bp, reverse 507–530 bp); SIRT6 (GenBank NM_016539; oligonucleotide forward 301–324 bp, reverse 487– 510 bp); SIRT7 (GenBank NM_016538; oligonucleotide forward 605–628 bp, reverse 849–872 bp). Amplification conditions were: denaturation 95uC, 30 sec; annealing 55uC, 1 min; extension 68uC,1 min. [a-32P]dATP was added to the reaction (0.04 mCi/ml); 32 cycles were performed to obtain sirtuin amplicons, whereas 24 cycles were used for GAPDH amplicon. PCR products were separated on a 6% polyacrylamide gel and quantitatively analyzed in a Typhoon Trio imager system using the IMAGE QUANT version 5.0

software (GE Healthcare, Amersham, UK). PCR products were also separated on a 2% agarose gel, extracted from the gel with the Gel Extraction kit (Qiagen) and sequenced.

4.4 Flow cytometry analysis

Keratinocyte expression of membrane Intercellular Adhesion Molecule (ICAM)-1, Human Leukocyte Antigen (HLA)-DR, and Major Histocompatibility Complex (MHC) Class I was evaluated using fluorescein isothiocyanate-(FITC)-conjugated anti-CD54 (84H10, Immunotech, Marseille, France), anti-HLA-DR (L243, BD Pharmingen, Franklin Lakes, NJ, USA), and anti-HLA-ABC (G46-2.6, BD Pharmingen) monoclonal antibodies (mAbs). In control samples, staining was performed using isotype-matched control Abs. Cells were analyzed with a FACScan equipped with Cell Quest software (Becton Dickinson, Mountain View, CA).

4.5 Western Blotting

Total proteins were prepared by solubilizing cells in RIPA buffer (1% NP-40, 0.5% sodium dehoxycholate, and 0.1% SDS in PBS containing a mixture of protease and phosphatase inhibitors). Alternatively, cytosolic and nuclear extracts were prepared from cells grown in 75-mm flasks, which were lysed with 1 ml cold Buffer A (20 mM HEPES [pH 7.9], 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 0.2% NP-40, and 1 mM DTT plus protease and phosphatase inhibitors). After centrifugation, the supernatants containing cytoplasms were collected, whereas the pellets containing nuclei were resuspended in 0.4 ml cold Buffer B (20 mM HEPES [pH 7.9], 0.35 M NaCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 10% glycerol, and 1 mM DTT plus protease and phosphatase inhibitors). After incubation at 4°C for 30 min, the suspensions were centrifuged at 14,000 rpm for 10 min, and the supernatants collected and diluted 5-fold in Buffer C (20 mM HEPES [pH 7.9], 60 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 5% glycerol, 0.05% NP-40, and 1 mM DTT plus protease and phosphatase inhibitors). The resulting samples were aliquoted and frozen at –80°C. Total, cytosolic, or nuclear proteins were subjected to SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The latter were blocked and probed with various primary Abs diluted in PBS containing 5% nonfat dried milk or 3% BSA. The primary Abs employed for the study were as follows: anti-PCNA (PC10), anti-cyclin D1 (DCS-6), anti-phospho-RB (Ser795), anti-lamin A/C (346), anti-α-tubulin (B-7), anti-β-actin (C-11), anti-phospho-STAT3 (Ser727), anti-STAT3 (C-20), anti-phospho-STAT1 (Tyr701), anti-STAT1 (E-23), antiphospho-ERK1/2 (E-4), anti-ERK1/2 (C16), and anti-SIRT1 (H-300), and HRP-

conjugated anti-c-myc (9E10) all provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-KRT1, anti-KRT5, anti-KRT14, and anti-loricrin Abs were from Covance (Emeryville, CA, USA), whereas anti-phospho-STAT3 (Tyr705) and anti-acetyl-STAT3 (K685) were from Cell Signaling Technology (Danvers, MA, USA). Anti-FLAG (M2; Sigma-Aldrich, Saint Louis, MO, USA) and anti-SIRT7 (RB1973; Abgent, San Diego, CA, USA) were also used. Western blotting filters were properly developed with anti-mouse, anti-goat, or anti-rabbit Ig Abs conjugated to HRP using the ECL-plus detection system (Amersham), or, otherwise, the SuperSignal West Femto kit (Pierce, Rockford, IL, USA).

4.6 Transient RNA interference

STAT3, SIRT1, SIRT2, SIRT5, SIRT7 and SOCS3 were knocked down by using a pool of 4 small short interfering (si)RNAs (ON-TARGET*plus* SMARTpool, Dharmacon RNA Technology, Lafayette, CO, USA). In parallel, a pool of 4 Non-targeting siRNAs was used as negative control. Primary cultures were transfected with STAT3, SIRT1, SIRT2, SIRT5, SIRT7, SOCS3 or irrelevant siRNA at 50 nM final concentration using Interferin reagent (Polyplus Transfection, New York, NY, USA). After 2 d of mRNA silencing, keratinocytes were treated with different stimuli.

4.7 Luciferase assay, transient and permanent transfections

Cultured keratinocytes grown in 12-well plates were transiently transfected with the STAT3-responsive plasmid pLucTKS3 (a generous gift of Prof. J. Turkson, University of Central Florida, Orlando, FL, USA) by using Fugene reagent (Promega, Madison, WI, USA). After transfection, cells were 8 hours stimulated with IL-22, and *Firefly* luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). pRL-null plasmid encoding the *Renilla* luciferase was included in each transfection. Primary keratinocytes were transiently transfected with pcDNA3-hSIRT1-FLAG (kindly provided by Prof. F. Ishikawa, Kyoto University, Kyoto, Japan), or pcDNA-myc/SOCS1-2-3 (a generous gift of Dr. A. Yoshimura, Kyushu University, Fukuoka, Japan) and pLucTKS3 luciferase was measured using the pcDNA3.1 plasmid as negative control. In addition, a panel of plasmids expressing wild-type or mutated SOCS3 were transiently co-transfected in keratinocytes with the pTKS3Luc plasmid, and luciferase activity was evaluated after IL-22 treatment. SOCS3 mutated plasmids dN25 (KIR-deleted), dN36 (KIR- and part of ESS-deleted), L22D (single-mutation in KIR), R71E (single-mutation in SH2) and dC40 (SOCS box-deleted) were a generous gift of Prof. Yoshimura. HaCaT cells were permanently transfected with pcDNA-myc/SOCS1-2-3 or empty pcDNA3 plasmids linearized by

ScaI restriction endonuclease (Boehringer Mannheim, Mannheim, Germany). Genetycin-resistant clones were selected after ~20 days by adding 0.4 mg/ml G418 (Invitrogen) to the culture medium. HaCaT clones expressing SOCS1-2 or -3 proteins were selected by Western blot analysis with the anti-c-myc Ab.

4.8 Cristal violet assay

Primary keratinocyte cells ($2x10^4$) were seeded in 96-well plates, and, the day after, starved in KBM. Culture stimulation with IL-22 was conducted either in the presence or absence of 10 μM Ex-527 or 40 μM Resveratrol (both from Sigma-Aldrich). SIRT1-silenced cells were also used in some experiments. Moreover, SOCS1-3 stable clones were seeded in 96-well plates ($1.5x10^4$ cells/well), and, the day after, starved in DMEM without serum. Culture stimulation with 70 ng/ml of IL-22 was conducted. After 2–4 d of treatment, cells were stained with 0.5% crystal violet, whose incorporation was measured at 540 nm in an ELISA reader (model 3550 UV ELISA reader; Bio-Rad, Hercules, CA, USA).

4.9 Scratch wound healing assays

Keratinocytes were STAT3 or SIRT1 grown at 100% of confluence, and then scratched with the tip of a p-200 pipette to create a uniform cell-free zone. Wounded monolayers were either incubated or not with 30 ng/ml IL-22. Microscopy pictures were taken with a digital camera at different time points following IL-22 treatment. The residual gap between migrating keratinocytes was measured with a computer-assisted image analysis system (Axiovision, Zeiss, Oberkochen, Germany), and expressed as percentage of the initial scratched area.

4.10 Immunohistochemistry

Cryostatic sections were incubated with monoclonal antibodies (mAbs) against SIRT1 (E104; Abcam, Cambridge, UK) and CD3 (BD-Pharmingen, Franklin Lakes, NJ, USA). Secondary biotinylated mAbs and staining kits (Vector Laboratories, Burlingame, CA, USA) were used to develop immunoreactivity. Figures depict one experiment that is representative of all the patients investigated.

5. Results (1)

5.1 IL-22 induces chemokines, antimicrobial peptides and proliferative molecules but inhibits differentiative markers in human keratinocytes.

IL-22 controls several biological processes in keratinocytes, including proliferation differentiation, migration, and production of molecules involved in the innate immune responses [26]. In particular, IL-22 was able to up-regulate mRNA expression of the chemokines CXCL8 and CXCL1 as well as the antimicrobial peptide HBD-2 and the S100A7, with significant levels of induction at late time points of stimulation (24–48 h; Fig. 5.1A). IL-22 also induced high amounts of SOCS3, which was rapidly induced (1 h), but did not affect SOCS1 mRNA expression. In parallel, it has been found that IL-22 could not regulate the membrane expression of ICAM-1, MHC class I and II molecules, induced by 36h of treatment with IFN-γ and TNF-α (Fig. 5.1B). Although IL-22 is considered a potent inducer of epidermal hyperplasia in pathological conditions, little information exists about its effect on the induction of molecules controlling keratinocyte proliferation. To this end, IL-22-treated keratinocytes were analyzed for the expression of proteins inducing cell cycle progression and, thus, essential for cell proliferation [59]. As shown in Fig. 5.1C, IL-22 maintained high nuclear levels of PCNA, cyclin D1 and pRB after 9 and 24 h, as compared to untreated cultures, which showed a decreased expression at these time points. In parallel, IL-22 could block the down-regulation of KRT5 and KRT14, two cytokeratins typical of proliferating keratinocytes, in cultures undergoing terminal differentiation (4 d of growth after confluence; Fig. 5.1D). *Vice versa*, IL-22 inhibited the expression of KRT1, a structural protein highly present in keratinocytes committed to terminal differentiation (Fig. 5.1D). IL-22 also potently decreased the protein accumulation of loricrin, which is a component of the cross-linked cell envelope in the uppermost layers of the epidermis (Fig. 5.1D).

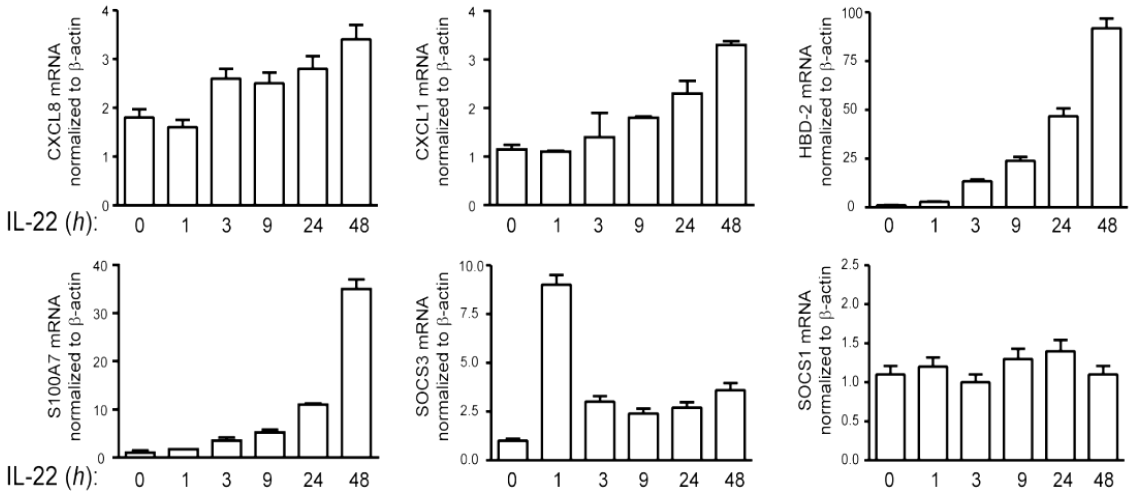

Figure 5.1A: IL-22-induces many inflammatory genes in human cultured keratinocytes. CXCL8, CXCL1, HBD-2, S100A7, SOCS3, and SOCS1 mRNA expression was evaluated by Real-time PCR analysis of mRNA from samples untreated or stimulated with IL-22 (50 ng/ml), and normalized to β-actin mRNA.

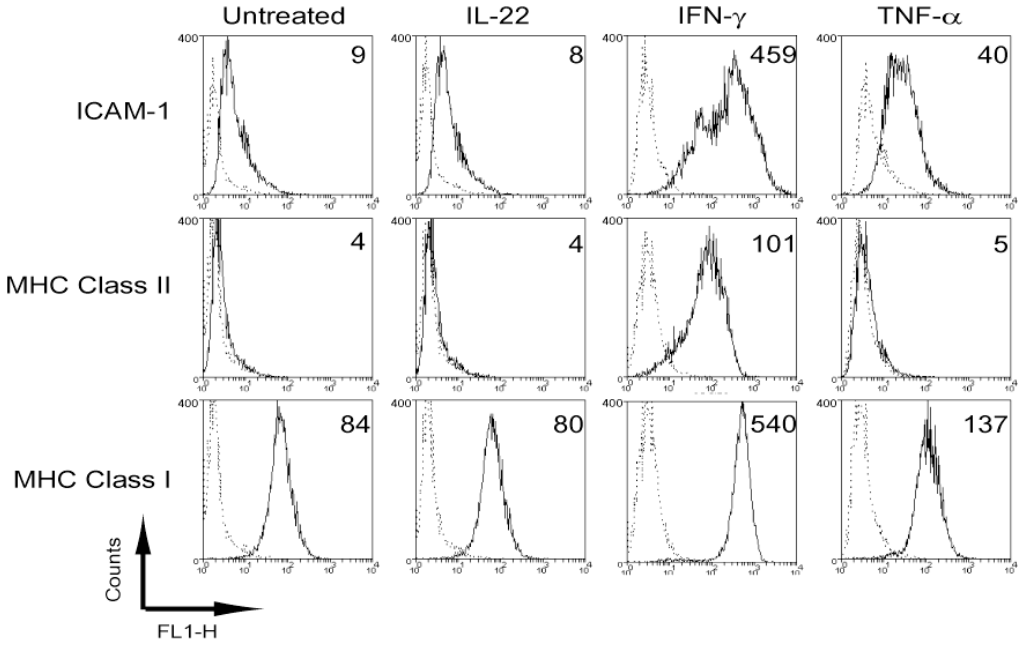

Figure 5.1B: IL-22 does not control the expression of immune-modulatory molecules in keratinocytes. Cytofluorymetric analysis of ICAM1, MHC Class I and Class II in 36 h stimulated culture keratinocytes with IL-22 (50 ng/ml), IFN-γ (200 U/ml) or TNF-α (50 ng/ml).

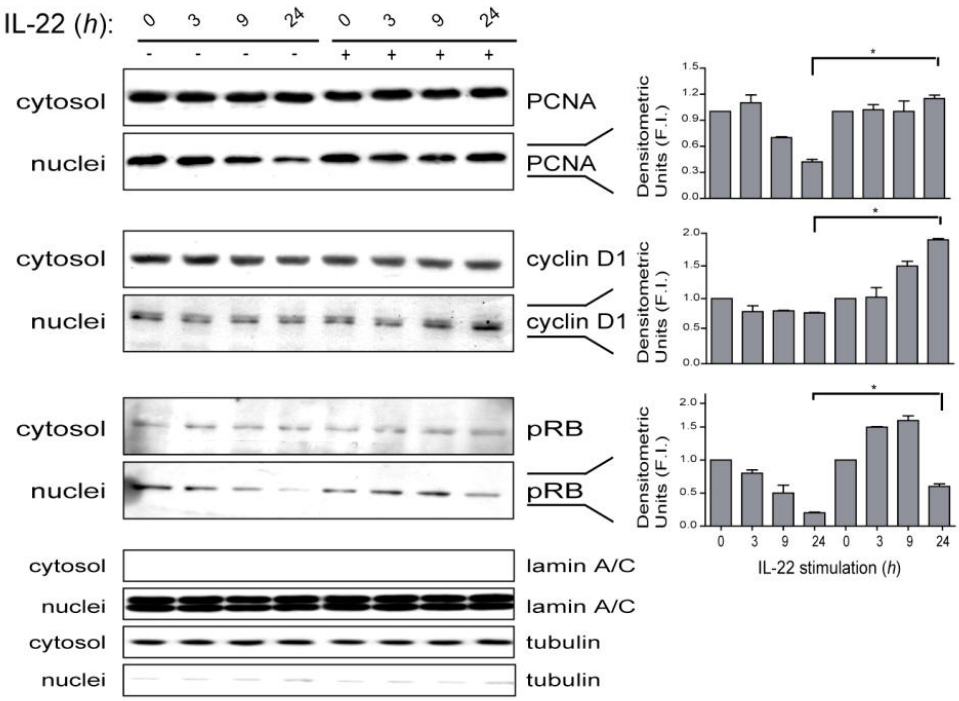

Figure 5.1C: IL-22 induces the nuclear translocation of PCNA, cyclin D1 and pRB proteins in human keratinocytes. PCNA, cyclin D1, and pRB levels were evaluated by Western blotting in cytosols or nuclei prepared from keratinocytes either treated or not with IL-22. Proper fractionation of nuclei and cytosols was verified by analyzing lamin A/C and tubulin expression, respectively. Graphs represent densitometric analyses of nuclear PCNA, cyclin D1, and pRB. Data are expressed as mean ± SD fold induction (F.I.), calculated relative to the untreated samples (*t*0), which were given a value of 1. **P* < 0.05.

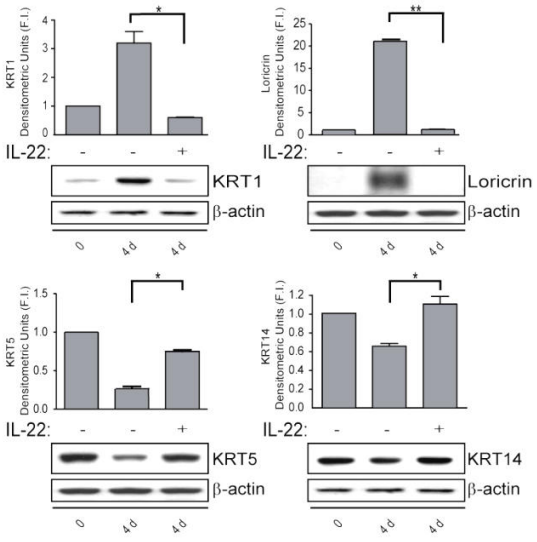

Figure 5.1D: IL-22 opposes the reduction of markers of keratinocyte proliferation whereas reduces markers of keratinocyte differentiation. Keratinocyte cultures were grown at 100% of confluence (*t*0) and left in culture for another 4 d in presence or absence of IL-22. Total lysates were analyzed by Western blotting using anti-KRT1, anti-KRT5, anti-KRT14, or anti-loricrin Abs. Graphs represent densitometric analyses of total KRT1, KRT5, KRT14, and loricrin normalized to β-actin signals. **P* <0.05; ***P* < 0.01.

5.2 STAT3 is the main mediator of IL-22-induced effects in human keratinocytes.

The analysis of IL-22-induced molecular cascades demonstrated that STAT3 signaling was rapidly induced in primary cultures of keratinocytes, and, in particular, STAT3 phosphorylation in Tyr705 was observed after 15–30 min of treatment with IL-22 (Fig. 5.2A). STAT3 phosphorylation in Ser727 residue, constitutively detected in keratinocytes, was also enhanced by IL-22, showing peculiar biphasic kinetics (Fig. 5.2A). IL-22 did not affect STAT1 activation but significantly induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation after 30 min of treatment (Fig. 5.2A). To determine whether STAT3 expression and activation influenced the IL-22-induced regulation of CXCL8, CXCL1, HBD-2, S100A7, SOCS3, and of the proteins associated to keratinocyte proliferation and differentiation, STAT3 mRNA silencing was performed in IL-22-activated or untreated keratinocyte cultures (Fig. 5.2B). Following silencing of STAT3 mRNA, keratinocytes were not able to up-regulate SOCS3 mRNA in response to IL-22 treatment, but continued to express HBD-2 and S100A7 (Fig. 5.2C). In parallel, CXCL8 and CXCL1 were only slightly induced by IL-22, indicating that STAT3 positively regulates these genes, but other mechanisms can control their expression. In addition, considering the delayed kinetics of CXCL8 and CXCL1 induction by IL-22, it is plausible that the STAT3 effect on these genes was indirect. Also, the nuclear accumulation of PCNA, cyclin D1, and pRB proteins observed after 24 h of incubation with IL-22 was significantly dependent on STAT3 expression (Fig. 5.2D). The negative effect of IL-22 on KRT1 expression did not occur when cells were STAT3 depleted (Fig. 5.2E). Similarly to KRT1, loricrin expression substantially increased in keratinocytes silenced for STAT3 (Fig. 5.2E), suggesting that STAT3 acts as a repressor of these molecules. On the contrary, the modulation of KRT5 and KRT14 expression by IL-22 treatment was independent from STAT3 (Fig. 5.2E). STAT3 mRNA and protein silencing by siRNA in keratinocytes was substantial and specific since STAT1 expression was unchanged (Fig. 5.2B). In summary, these data indicated that the majority of the IL-22-induced effects in human keratinocytes were mediated by STAT3 signaling.

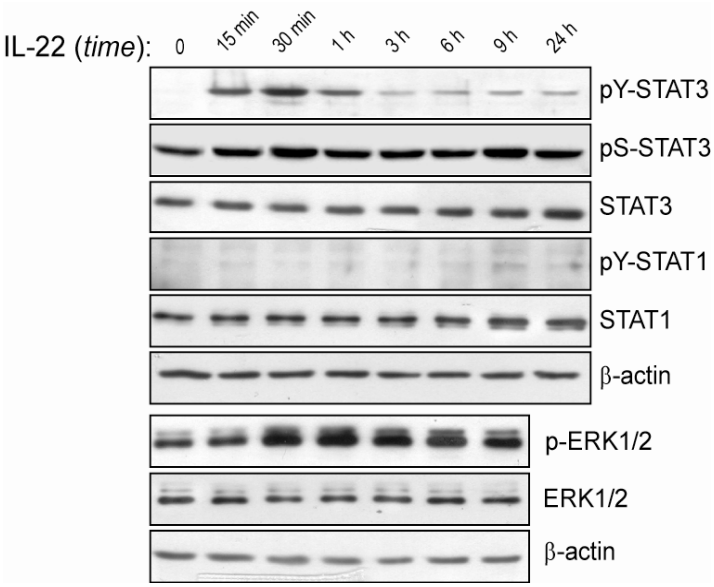

Figure 5.2A: In keratinocytes IL-22 induces STAT3 and ERK1/2 activation. Total STAT3, STAT3 phosphorylated in Tyr705 and in Ser727, total STAT1, STAT1 phosphorylated in Tyr701, total ERK1/2, and ERK1/2 phosphorylated in Tyr204 were detected by Western blotting. Immunoblot stainings are representative of 3 independent experiments performed on 3 different keratinocyte strains.

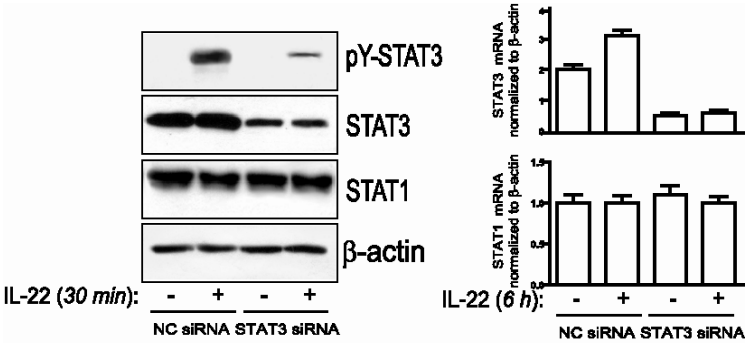

Figure 5.2B: STAT3 transient silencing in cultured keratinocytes. Keratinocyte cultures were transfected with siRNA specific for STAT3 or irrelevant siRNA (NC). After 48 h transfection, cells were stimulated with IL-22 or not for the indicated time periods. Analyses of STAT3 and STAT1 protein accumulation or mRNA expression were performed by Western blotting or real-time PCR, respectively.

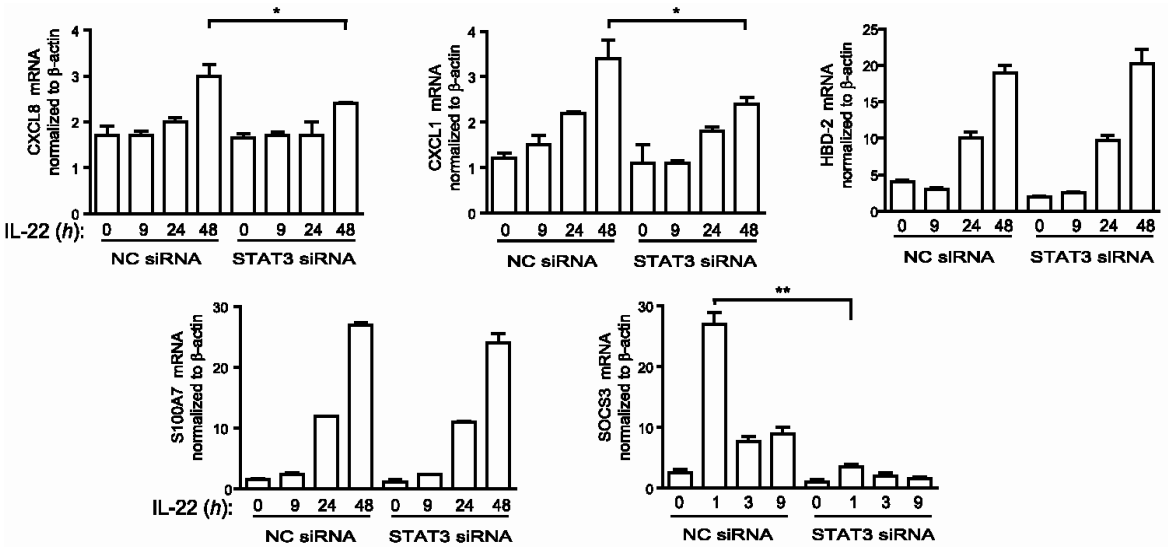

Figure 5.2C: SOCS3, and at less extent, CXCL1 and CXCL8, are induced by IL-22 treatment in a STAT3-dependent manner. CXCL8, CXCL1, HBD-2, S100A7, and SOCS3 mRNA expression was analyzed in samples silenced for STAT3 or treated with NC siRNA and then stimulated with IL-22 as indicated. **P* < 0.05; ***P* < 0.01.

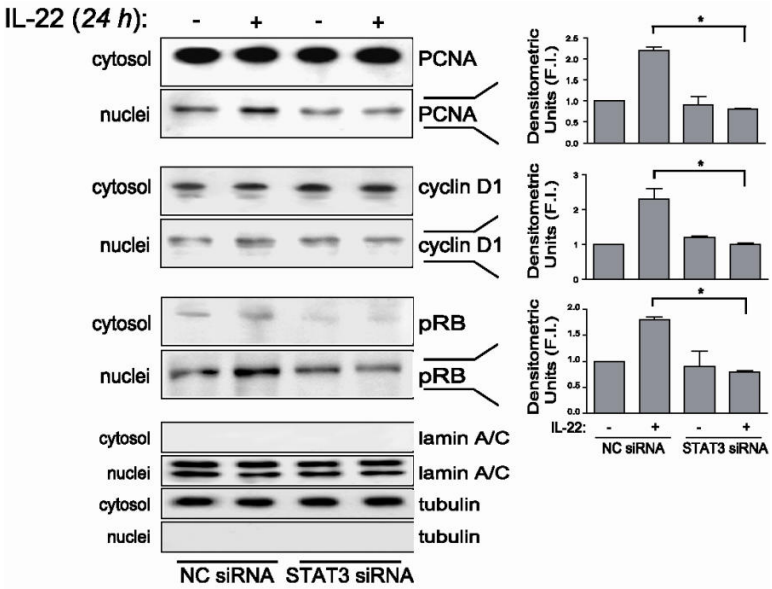

Figure 5.2D: The IL-22-triggered nuclear translocation of proliferative markers is a STAT3-dependent event in keratinocytes. Western blotting of nuclei and cytosols served to study PCNA, cyclin D1, and pRB protein accumulation in untreated or IL-22-stimulated keratinocytes transfected with irrelevant and STAT3-specific siRNA. Graphs represent densitometric analyses of nuclear PCNA, cyclin D1, and pRB. Western blots are representative of 3 independent experiments performed on 3 different keratinocyte strains. **P* < 0.05.

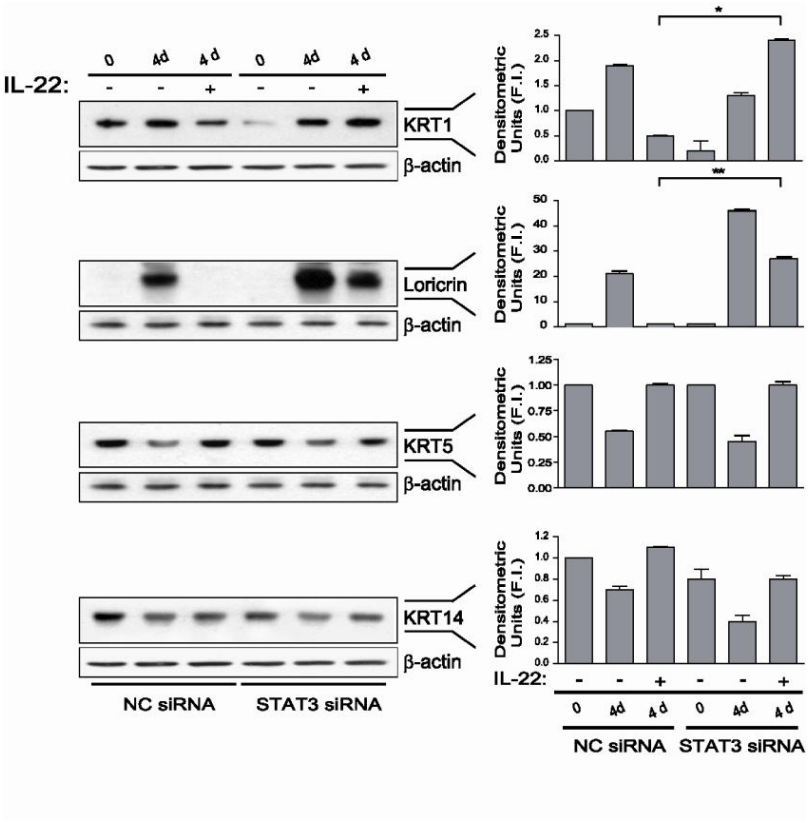

Figure 5.2E: The IL-22-derived reduction of differentiative markers in keratinocytes is STAT3-dependent. STAT3-silenced keratinocyte cultures undergoing terminal differentiation (4 *d*) either activated or not with IL-22 were studied for KRT1, KRT5, KRT14, and loricrin expression by Western blotting. Graphs show densitometric values of KRT1, KRT5, KRT14, and loricrin. Western blots are representative of 3 independent experiments performed on 3 different keratinocyte strains. **P* < 0.05; ***P* < 0.01.

5.3 SIRT1 inhibits STAT3 activation by reducing its Lys685 acetylation.

STAT3 is tightly regulated at the post-translational level, and, in particular, its acetylation in Lys685 is indispensable for the phosphorylation in Tyr705 and trans-activating function [30]. Lys685 acetylation of STAT3 can be, in turn, inhibited by SIRT1, which is also known to reduce proliferation and promote terminal differentiation of epidermal keratinocytes [39, 42]. Therefore, we supposed that SIRT1 and/or other sirtuin members could oppose IL-22 signaling and effects on keratinocytes by inhibiting STAT3 acetylation in Lys685 and, thus, STAT3 phosphorylation and function. We firstly analyzed the expression of the seven sirtuin members in cultured healthy keratinocytes. Semi-quantitative PCR analysis demonstrated that keratinocytes constitutively expressed substantial levels of SIRT1, SIRT2,

SIRT5 and SIRT7 mRNA, and very low or null levels of SIRT3, SIRT4 and SIRT6 mRNA (Fig. 5.3.A). The manipulation of SIRT1-2-5 and -7 by transient RNA interference demonstrated that only SIRT1 could significantly up-regulate STAT3 activation. In fact, SIRT1- but not SIRT2- or SIRT5- or SIRT7-depleted keratinocytes showed higher levels of phosphorylated STAT3 (Tyr 705) in response to IL-22 compared to control cells (Fig. 5.3B). Conversely, SIRT1 depletion did not affect the phosphorylation in Ser727 of STAT3 nor the activation of STAT1 and ERK1/2 (Fig. 5.3C). In addition, the analysis of STAT3 activity in IL-22-treated SIRT1-silenced keratinocytes transfected with a STAT3-responsive plasmid, pLucTKS3, revealed that SIRT1 negatively regulates STAT3 activity (Fig. 5.3D, Left graph). On the contrary, SIRT1 overexpression by transfection with a human SIRT1-encoding plasmid dose-dependently inhibited the pTKS3 luciferase activity (Fig. 5.3D, Right graph). Since SIRT1 opposed IL-22 effects by counteracting STAT3 phosphorylation in Tyr705, the capability of SIRT1 to modulate STAT3 acetylation was also studied. Firstly, STAT3 acetylation in Lys685 was analyzed by Western blotting in keratinocytes untreated or activated with IL-22. STAT3 acetylation was constitutive and not modulated by IL-22 (Fig. 5.3E). When keratinocytes were depleted of SIRT1, Lys685 constitutive acetylation of STAT3 was significantly enhanced compared to control cells, as assessed using different keratinocyte strains (n=3) and in independent experiments of silencing (3 representative experiments are shown in Fig. 5.3F). The effect of SIRT1 was specific since SIRT7 did not influence STAT3 acetylation in Lys685 (Fig. 5.3F). Collectively, these data demonstrated that SIRT1 negatively modulates STAT3 capability to control the expression of molecules regulated by IL-22 by inhibiting Lys685 acetylation of STAT3 and, thus, its phosphorylation in Tyr705.

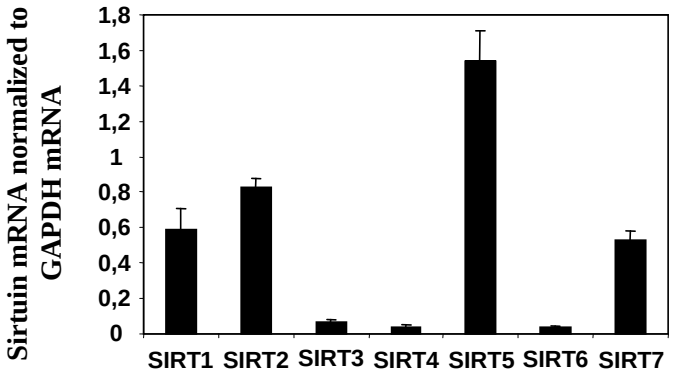

Figure 5.3A: Primary cultures of human keratinocytes highly express SIRT1-2-5 and -7 mRNA, and low or null levels of SIRT3-4 and -6. SIRT1-SIRT7 mRNA expression was evaluated in untreated cultured keratinocytes by semi-quantitative PCR analysis.

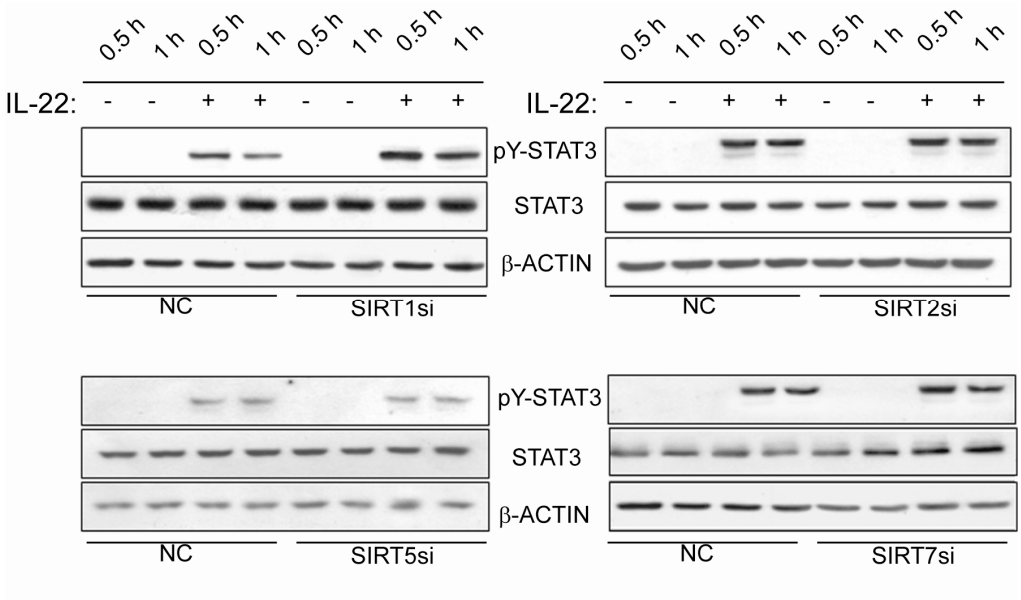

Figure 5.3B: Among sirtuins only SIRT1 reduces the IL-22-derived Tyr705 phosphorylation of STAT3. Keratinocyte cultures were transfected with SIRT1-, SIRT2-, SIRT5-, SIRT7-specific or irrelevant siRNA. After 48-h transfection, cells were stimulated with IL-22 or not, as indicated. Analyses of STAT3 phosphorylated in Tyr705 (pY-STAT3) and total STAT3 were performed by Western blotting.

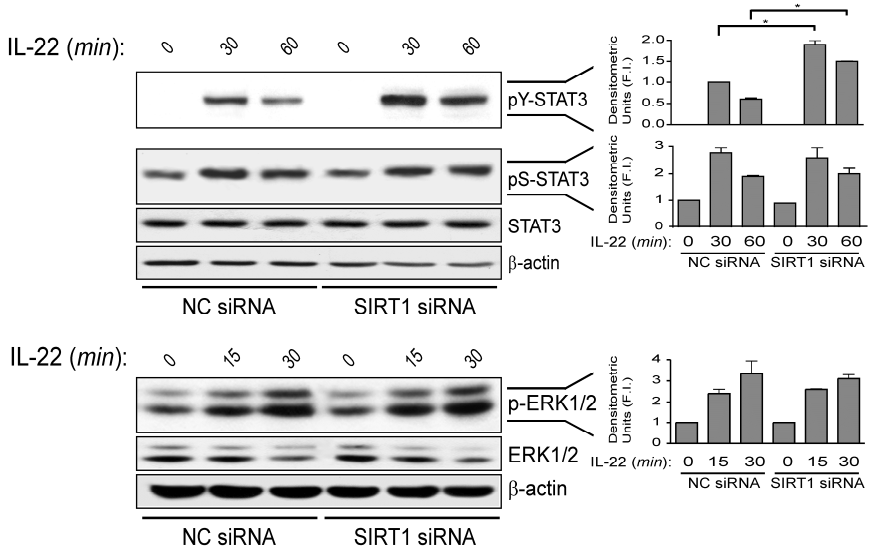

Figure 5.3C: SIRT1 does not influence the IL-22-induced STAT3 Ser727 and ERK1/2 Tyr204 phosphorylation. Keratinocyte cultures were transfected with SIRT1-specific or irrelevant siRNA. After 48 h transfection, cells were stimulated with IL-22 or not, as indicated. Analyses of STAT3 phosphorylated in Tyr705 (pY-STAT3) and in Ser727 (pS-STAT3), total STAT3, total ERK1/2, and ERK1/2 phosphorylated in Tyr204 were performed by Western blotting. Densitometries of blots relative to pY-STAT3, pS-STAT3, and phosphorylated ERK1/2 are shown.

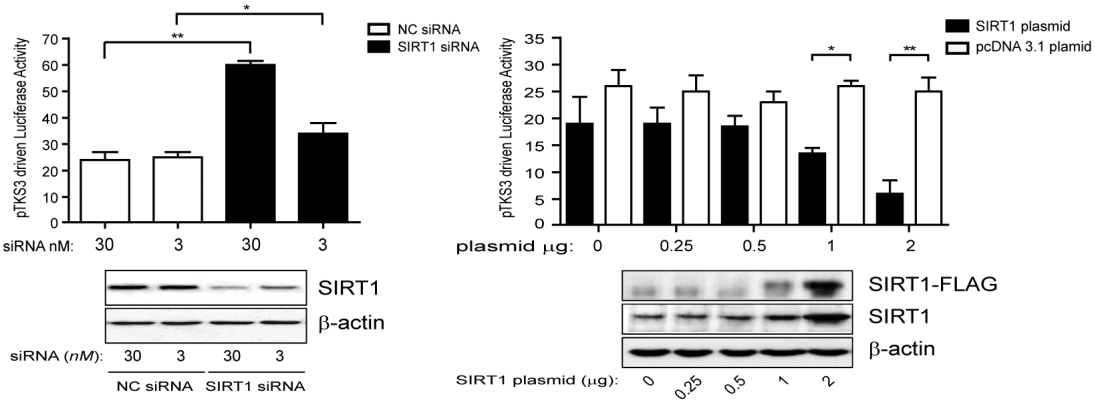

Figure 5.3D: IL-22-induced STAT3 activity is counterregulated by SIRT1. *Left graph.* Keratinocytes were treated with irrelevant siRNA or with siRNA specific for SIRT1, at 3 or 30 nM concentrations. Cells were then transiently transfected with pTKS3-Luc reporter plasmid and stimulated with IL-22. Effects of SIRT1 depletion on the STAT3-responsive plasmid were evaluated by assaying luciferase activity. *Right graph.* Cultured keratinocytes were cotransfected with increasing amounts (0–2 μg/well) of pcDNA3.1 (open bars) or pcDNA3-hSIRT1-FLAG (solid bars), and of 0.5 μg pTKS3-Luc reporter plasmid; 3 independent experiments. Data are expressed as means ± SD of *Firefly* luciferase values normalized to *Renilla* luciferase and micrograms of total proteins. **P*<0.05; ***P* <0.01.

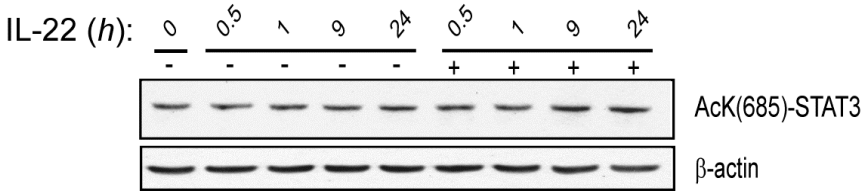

Figure 5.3E: In keratinocytes STAT3 is constitutively acetylated in Lys685 residue. STAT3 acetylation in Lys685 [AcK(685)-STAT3] was analyzed by Western blotting performed on untreated or IL-22-activated keratinocytes. Blots are representative of 3 independent experiments.

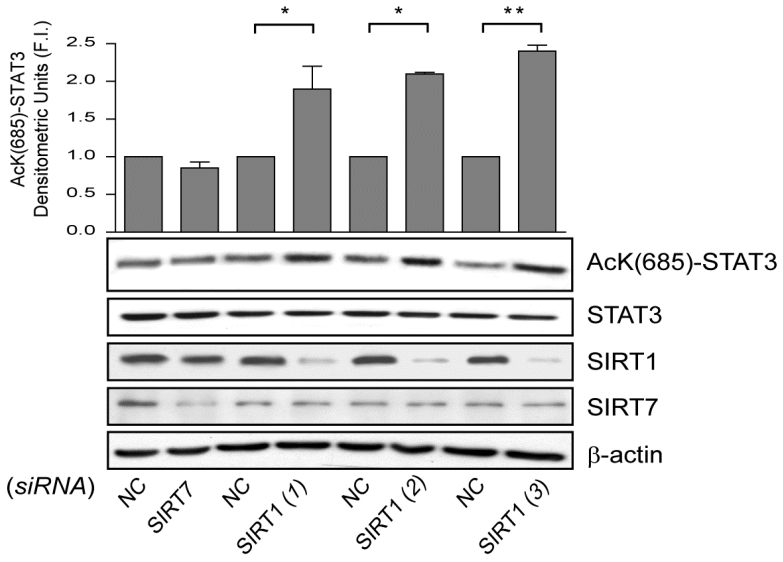

Figure 5.3F: SIRT1 deacetylates STAT3 in Lys685 in keratinocytes. STAT3 acetylation in Lys685 was analyzed by Western blotting by using lysates from keratinocytes transfected with siRNAs specific for SIRT1 or SIRT7, and compared to NC. Results are representative of 6 independent experiments. Densitometry of blot relative to AcK(685)-STAT3 is shown. **P* <0.05; ***P* <0.01

5.4 IL-22-induced STAT3-dependent gene expression as well as proliferative and migratory processes in keratinocytes are counteracted by SIRT1.

To further clarify the role of SIRT1 in the control of IL-22-induced STAT3-dependent gene expression in keratinocytes, we manipulated SIRT1 mRNA expression through RNA interference. We found that SIRT1 silencing in IL-22-treated keratinocytes strongly up-regulated SOCS3 mRNA expression (Fig. 5.4A). CXCL8 and CXCL1 expression also increased in SIRT1-depleted keratinocytes compared to control cells (Fig. 5.4A). As expected, HBD-2 and S100A7 gene expression, which were not STAT3-dependent, were not influenced by SIRT1 silencing (Fig. 5.4A). Consistently, SIRT1-depletion enhanced the positive effect of IL-22 on PCNA, cyclin D1 and pRB nuclear translocation, which occurred in a STAT3-dependent manner (Fig. 5.4B). SIRT1-silencing also determined a marked reduction of KRT1 and loricrin expression in keratinocytes undergoing differentiation, in agreement with previous findings [42] (Fig. 5.4C). Of note, SIRT1 depletion reinforced the inhibitory effect of IL-22 on KRT1 expression (Fig. 5.4C). Finally, KRT5 and KRT14 up-regulation by IL-22 in keratinocytes undergoing differentiation *in vitro*, an event found to be independent from STAT3 (Fig. 5.2E), was not affected by SIRT1 silencing (Fig. 5.4C). To clarify the role of SIRT1 in the control of IL-22-induced keratinocyte proliferation, a functional *in vitro* assay measuring the keratinocyte proliferative rate was performed after the manipulation of SIRT1 function and expression by using chemical modulators of SIRT1 or through RNA interference, respectively. Crystal violet tests on keratinocyte cultures treated with Ex-527, a specific inhibitor of SIRT1 enzymatic activity, or with resveratrol, a polyphenol stimulating SIRT1 deacetylase function, showed that SIRT1 significantly opposed IL-22-induced keratinocyte proliferation (Fig. 5.4D, Left graph). Consistently, SIRT1-silenced keratinocytes proliferated more actively in response to IL-22 compared to cells transfected with irrelevant siRNA (Fig. 5.4D, Right graph). It has previously reported that IL-22 promotes wound healing in an *in vitro* injury model [11]. Therefore, SIRT1 ability to counteract IL-22 also in this system was studied, and it has been found that SIRT1 silencing in keratinocytes greatly enhanced IL-22-induced wound healing 2d after scratching (Fig. 5.4E). Since the IL-22-induced closure of keratinocyte layer was strictly STAT3-dependent, as assessed by mRNA interference of STAT3 (Fig. 5.4E), it is likely that the negative effects of SIRT1 on IL-22-induced wound healing occurred through STAT3 inhibition.

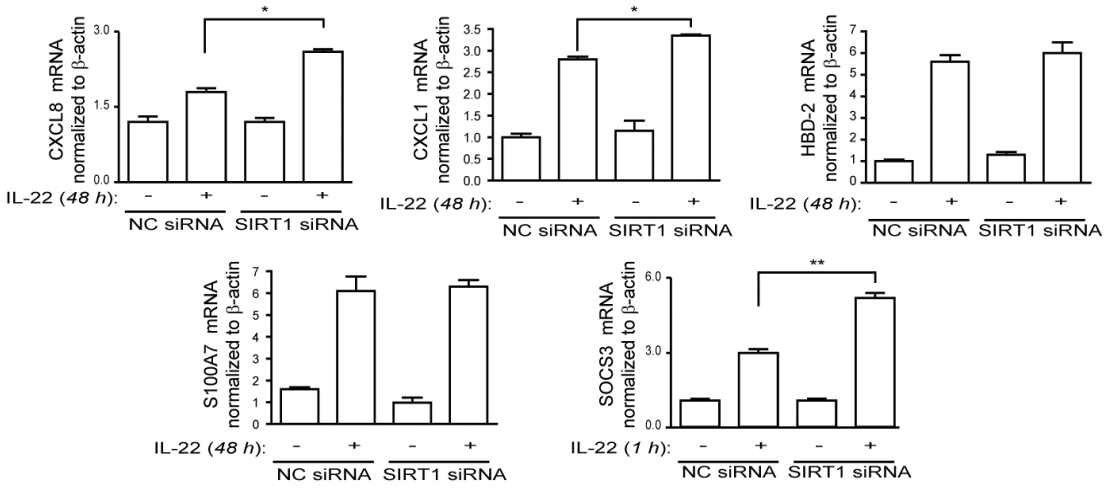

Figure 5.4A: SIRT1 counteracts the IL-22-derived and STAT3-dependent expression of SOCS3, CXCL1 and CXL8. Keratinocyte cultures were transfected with SIRT1-specific or irrelevant siRNA, and then stimulated with IL-22 or not, as indicated. CXCL8, CXCL1, HBD-2, S100A7, and SOCS3 mRNA expression was analyzed by real-time PCR. **P* <0.05; ***P* <0.01.

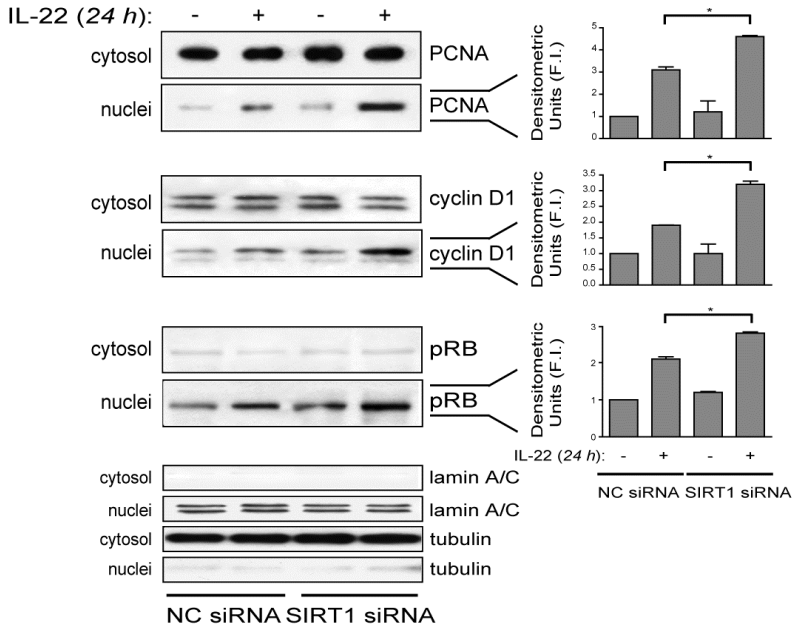

Figure 5.4B: SIRT1 inhibits the IL-22-derived and STAT3-dependent nuclear translocation of proliferative markers. Keratinocyte cultures were transfected with SIRT1-specific or irrelevant siRNA, and, then, 24-h stimulated with IL-22 or not. PCNA, cyclin D1, and pRB were analyzed by Western blotting. Graphs show densitometric values of PCNA, cyclin D1 and pRB staining on blots. Western blots are representative of 3 independent experiments performed on 3 different keratinocyte strains. **P* < 0.05.

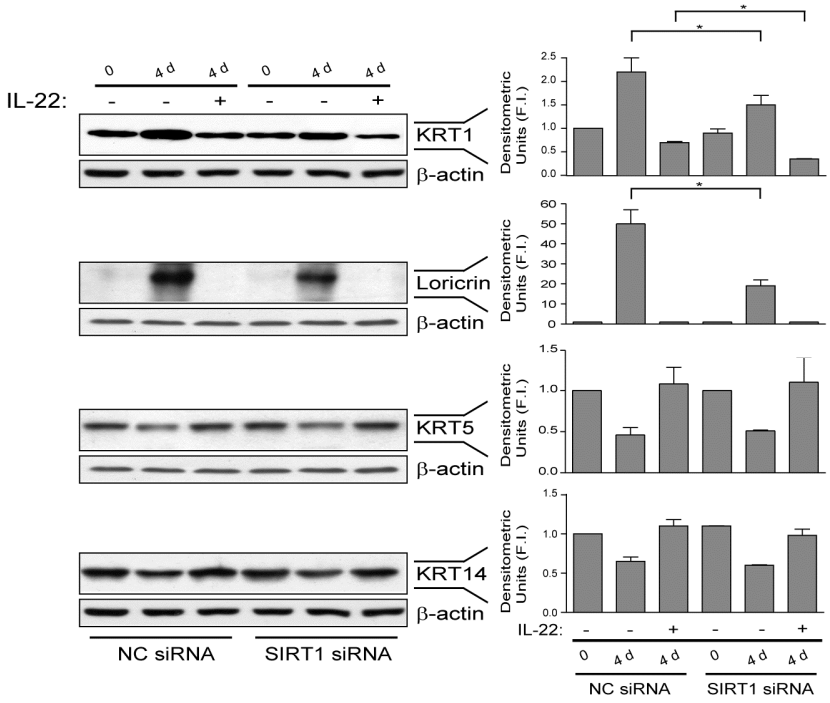

Figure 5.4C: SIRT1 counteracts the IL-22-induced and STAT3-dependent inhibition of differentiative marker expression. Keratinocyte cultures were transfected with SIRT1-specific or irrelevant siRNA, and, then, 4-d stimulated with IL-22 or not. KRT1, Loricrin, KRT5 and KRT14 were analyzed by Western blotting. **P* < 0.05.

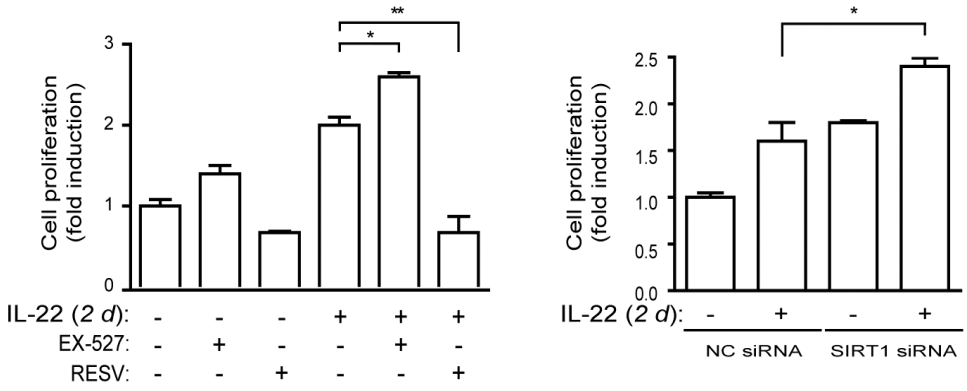

Figure 5.4D: Positive effect of IL-22 on keratinocyte proliferation is counteracted by SIRT1. Proliferation of keratinocytes treated with 10 μM Ex-527 or 40 μM resveratrol either in presence or absence of IL-22 was proportional to crystal violet incorporation (Left graph). Crystal violet assay was also performed on keratinocytes transfected with SIRT1 or irrelevant siRNA and treated with IL-22 (Right graph). Crystal violet incorporation was measured with an ELISA reader after 2 d of culture and is expressed as fold induction of treated *vs*. untreated samples, which were given a value of 1. **P* < 0.05; ***P* < 0.01.

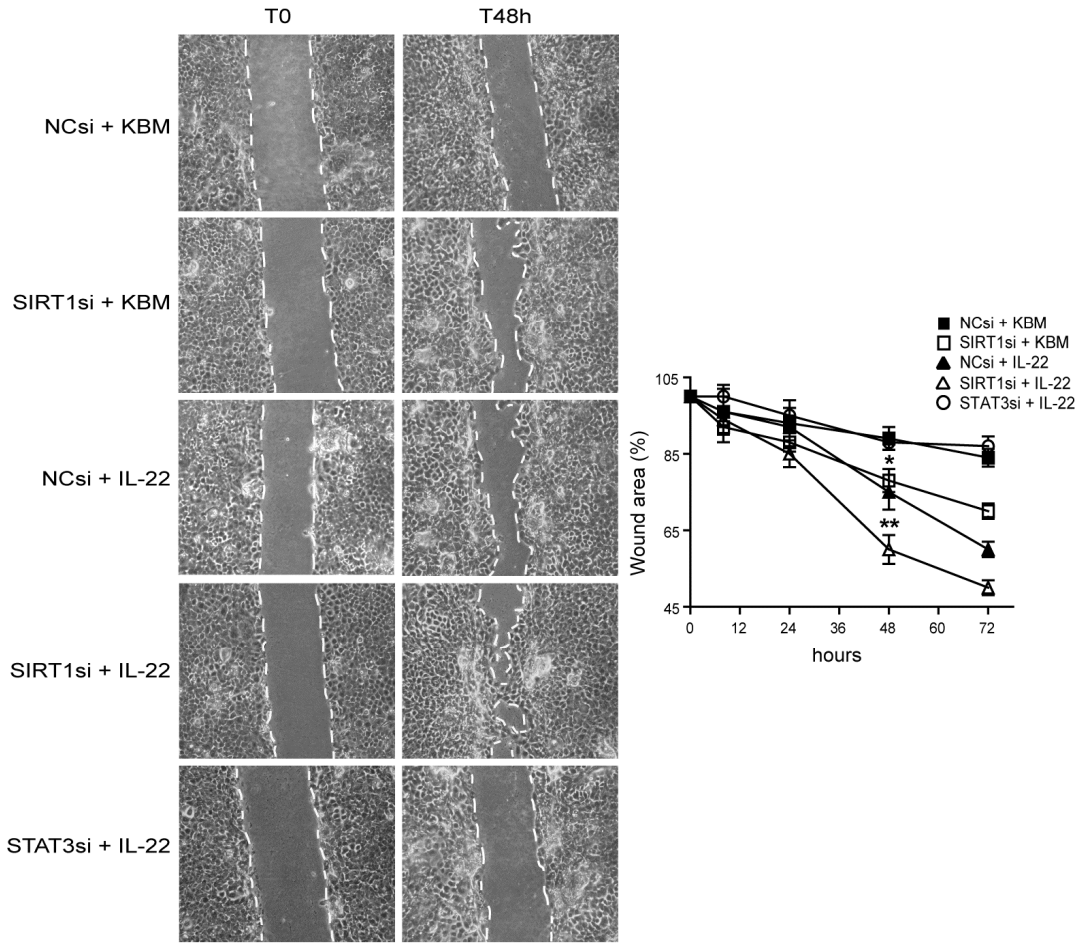

Figure 5.4E: Positive effect of IL-22 on keratinocyte migration is counteracted by SIRT1. Scratch assays were carried out on keratinocytes silenced for STAT3 (STAT3si) or SIRT1 (SIRT1si) or treated with irrelevant siRNA (NCsi), and incubated or not with 30 ng/ml IL-22 for 48 h. Residual gap between migrating keratinocytes is expressed as percentage of initial scratched area. **P* <0.01 *vs.* NCsi samples; ***P* < 0.01 *vs.* IL-22-treated NCsi samples.

5.5 SIRT1 expression in keratinocytes is down-regulated in psoriatic skin and, *in vitro*, after IFN-γ exposure.

STAT3 activity is enhanced in skin affected by psoriasis, especially in response to IL-22, and is responsible for the hyperproliferation and altered differentiation of the psoriatic epidermis [5]. Since SIRT1 efficiently controls IL-22-induced activity of STAT3 in keratinocytes, it has been investigated if SIRT1-STAT3 interplay in psoriasis could be deregulated. Therefore, SIRT1 expression levels were first analyzed in keratinocyte strains obtained from patients with psoriasis, and compared them with keratinocytes isolated from skin of healthy individuals. Keratinocyte cultures were prepared from LS and NLS skin biopsies obtained from the same donor. As shown in Figure 5.5A, patients with psoriasis and healthy donors showed similar SIRT1 levels, mainly localized in the cytosol, as assessed by Western blotting. Immunohistochemistry analysis confirmed that SIRT1 was cytosolic and uniformly expressed in keratinocytes of the basal layer of the epidermis (Fig. 5.5B*i–iii*). The pattern of SIRT1 expression was identical in healthy and NLS psoriatic skin (Fig. 5.5B*i*, *ii*), which showed also few infiltrating CD3+ T cells (Fig. 5.5B*iv*, *v*). Conversely, basal keratinocytes of LS psoriasis exhibited a reduced and less intense SIRT1 staining if compared to healthy and NLS skin, especially at the tips of the epidermal papillae close to the CD3+ T cell infiltrate (Fig. 5.5B*iii*, *vi*). Since T cells infiltrating psoriatic lesions produce very high amounts of IFN-γ (*i.e.*, Th1, Th1/Th17 and T cytotoxic cells), it has been hypothesized that SIRT1 decrement in LS psoriatic keratinocytes could be due to local T-cell-derived IFN-γ. Indeed, treatment of keratinocyte cultures with supernatants obtained from Th1 clones releasing high amounts of IFN-γ (25–35 ng/ml/10^6 cells) reduced SIRT1 protein expression (Fig. 5.5C). Keratinocyte treatments with recombinant cytokines typically present in psoriatic skin confirmed that only recombinant IFN-γ, and not TNF-α, IL-17, or IL-22, could reduce SIRT1 mRNA and protein expression in human keratinocytes (Fig. 5.5C). As a whole, these data demonstrate that although SIRT1 is expressed at comparable levels in keratinocytes from patients with psoriasis and healthy individuals, it is down-regulated in psoriatic epidermis, likely as a result of the local overexpression of IFN-γ.

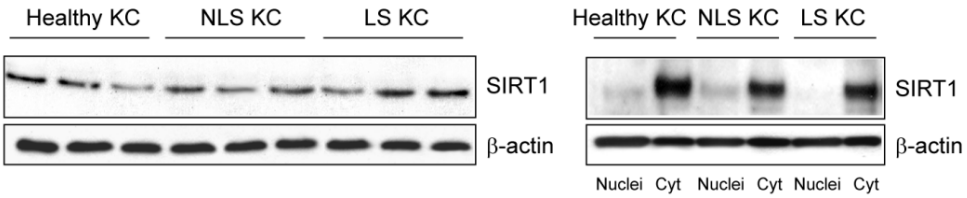

Figure 5.5A: SIRT1 *in vitro* expression is cytosolic and it is comparable in healthy and psoriatic keratinocytes. Cultured keratinocytes were prepared from healthy skin or from biopsies taken from uninvolved (NLS KC) or involved (LS KC) psoriatic skin. SIRT1 protein level was detected in total (Left panels) or fractionated (Right panels) lysates of keratinocytes; 3 of 6 representative psoriatic and healthy keratinocyte strains are shown.

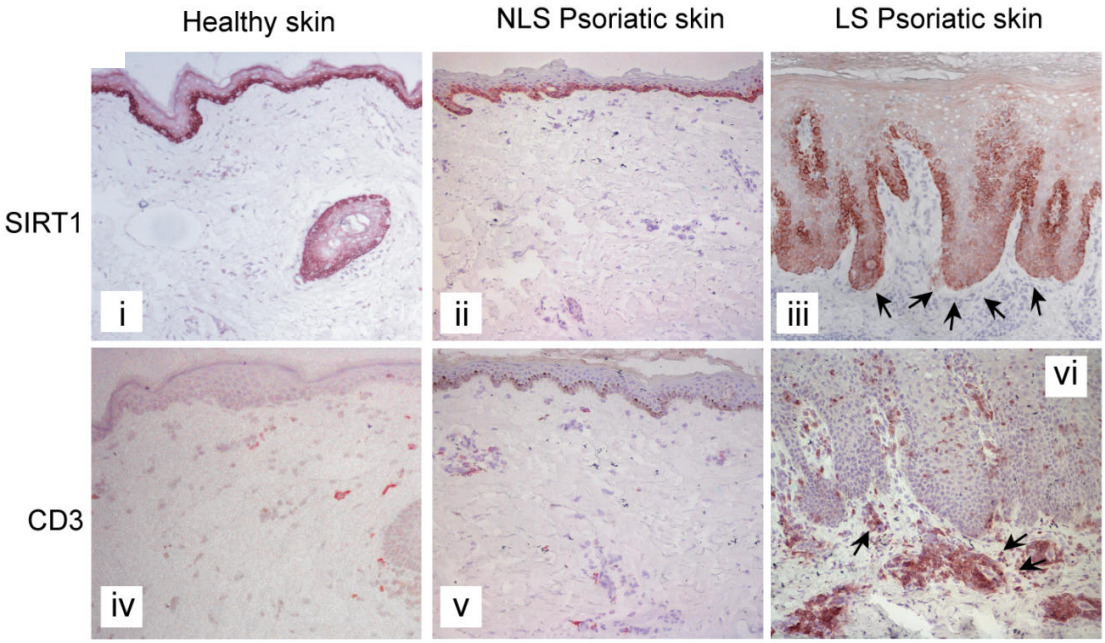

Figure 5.5B: SIRT1 *in vivo* expression is reduced in psoriatic epidermal papillae close to the Th1 lymphocytes. Immunohistochemistry for SIRT1 and CD3 (both stained in red) was performed on frozen sections from biopsies of psoriatic skin including LS (*ii*, *v*), and NLS (*iii*, *vi*) areas of plaques. Healthy skin was also analyzed (*i*, *iv*). Immunoreactivity was revealed by using 3-amino-9-ethylcarbazole as substrate. Arrows (*iii*) indicate area of epidermal papillae where SIRT1 staining was reduced. Representative stainings of 6 psoriatic sections analyzed are shown.

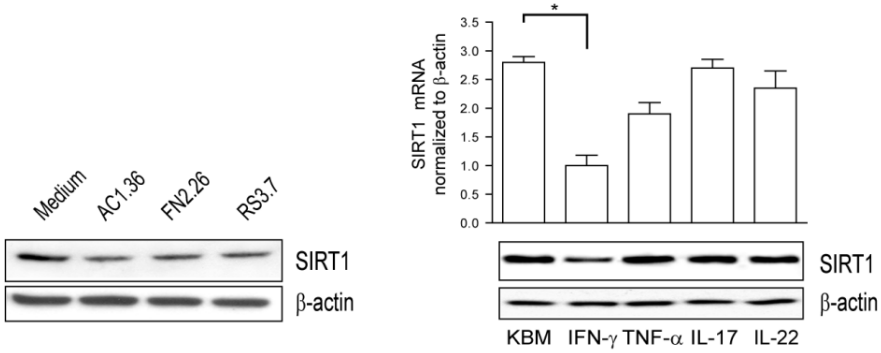

Figure 5.5C: IFN-γ reduces the SIRT1 expression levels. Western blotting of SIRT1 performed on keratinocyte cultures after 24 h activation with diluted (1:3) supernatants derived from 3 Th1 clones (AC1.36, FN2.26, and RS3.7) that produce high amounts of IFN-γ (25–35 ng/ml/10^6 cells). Th1 clones were prepared from peripheral blood of patients with psoriasis; and supernatants were collected after 48 h activation with anti-CD3 and anti-CD28. Alternatively, SIRT1 expression was analyzed by real-time PCR or Western blotting in samples activated with recombinant IFN-γ, TNF-α, IL-17 or IL-22 (right panels). Western blots are representative of 2 independent experiments performed on 2 different keratinocyte strains. *$P < 0.01$.

5.6 IFN-γ up-regulates basal STAT3 acetylation, thus reinforcing the IL-22-induced STAT3 phosphorylation and downstream effects in keratinocytes.

To see whether the reduced SIRT1 expression by IFN-γ could result in an increase of STAT3 acetylation and, thus, in enhanced STAT3 phosphorylation in response to IL-22, Western blotting experiments were performed on keratinocyte cultures prestimulated with IFN-γ or TNF-α, and then activated with IL-22. As shown in Fig. 5.6A, IFN-γ significantly enhanced basal STAT3 acetylation in Lys685 (lane 2 *vs.* 1) as well as the IL-22-induced phosphorylation of STAT3 in Tyr705, as compared to treatment with IL-22 alone (lane 5 *vs.* 4). The effect was specific for IFN-γ since TNF-α could not regulate neither basal STAT3 acetylation (Fig. 5.6 A, lane 3 *vs.* 1) nor IL-22-induced STAT3 phosphorylation (Fig. 5.6A, lane 6 *vs.* 4). Accordingly, SIRT1 silencing in keratinocytes further enhanced the IFN-γ-induced acetylation in Lys685 of STAT3 (Fig. 5.6A). Concomitantly to the up-regulation of IL-22-induced STAT3 phosphorylation, IFN-γ strengthened IL-22-promoted downstream effects. In particular, IFN-γ significantly enhanced IL-22-induced expression of CXCL8, CXCL1, and SOCS3 mRNA (Fig. 5.6B), as well as IL-22-inhibited accumulation of KRT1 (Fig. 5.6C). Interestingly,

nuclear PCNA was strongly reduced by IFN-γ (Fig. 5.6D), which notoriously exerts an antiproliferative effect on keratinocytes [60].

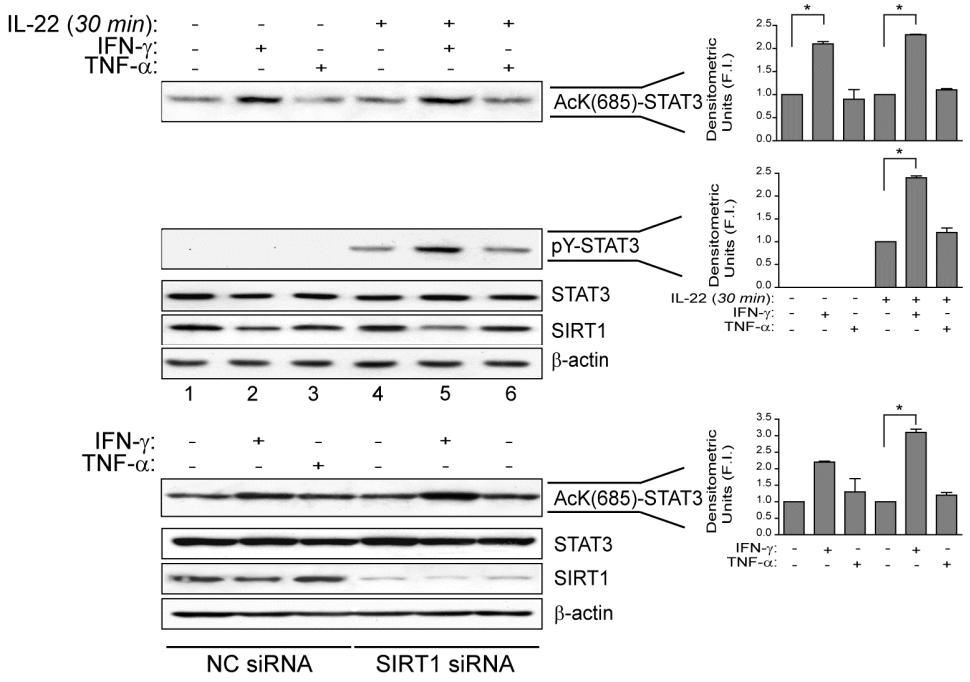

Figure 5.6A: IFN-γ up-regulates basal STAT3 Lys685 acetylation. Cultured keratinocytes were pretreated for 24 h with IFN-γ or TNF-α, then stimulated with IL-22 for 30 min. Total lysates were analyzed by Western blotting; amounts of acetyl STAT3 and phosphorylated STAT3 were analyzed by densitometry. Analyses of acetyl STAT3 were also performed on keratinocytes transfected with irrelevant or SIRT1 siRNA and activated for 24 h with IFN-γ or TNF-α. *$P < 0.01$.

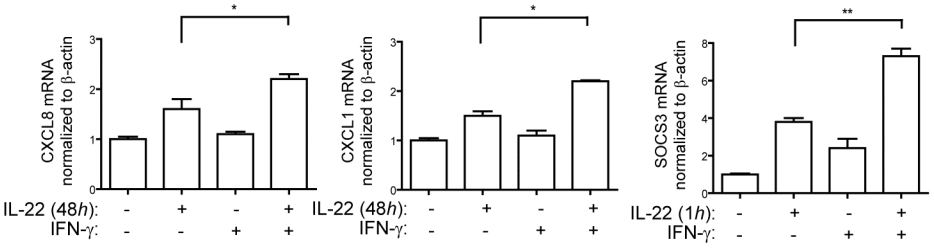

Figure 5.6B: IFN-γ reinforces the IL-22-triggered inflammatory gene expression. CXCL8, CXCL1, and SOCS3 mRNA expression was evaluated by real-time PCR in cultured keratinocytes 24h pre-treated with IFN-γ and then stimulated with IL-22 for indicated time. *$P < 0.05$; **$P < 0.01$.

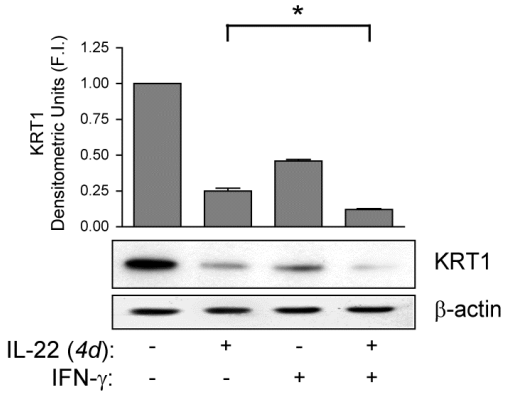

Figure 5.6C: IFN-γ potentiates the IL-22-derived KRT1 reduction. KRT1 expression analysis was performed on keratinocyte cultures undergoing terminal differentiation or stimulated with IL-22 and/or IFN-γ for 4 d. Variations of KRT1 levels were also determined by densitometry. Western blots are representative of 2 independent experiments performed on 2 different keratinocyte strains. **P* <0.05.

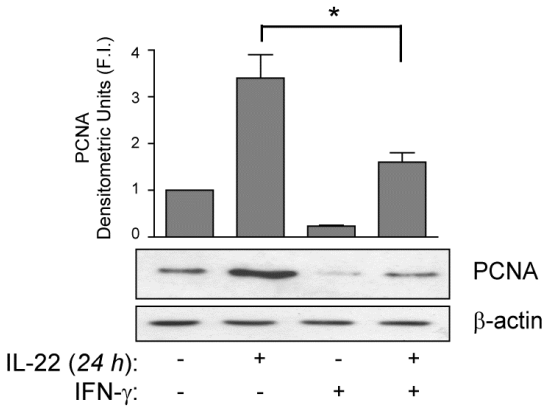

Figure 5.6D: IFN-γ has anti-proliferative effects in keratinocytes. Nuclear PCNA was analyzed in keratinocytes treated with IL-22 and/or IFN-γ for 24 h. Western blots are representative of 2 independent experiments performed on 2 different keratinocyte strains. **P* <0.05.

6. Results (2)

6.1 SOCS3 is induced by IL-22 and inhibits the cytokine-dependent STAT3 activation in keratinocytes.

It has previously reported that IL-22 is able to induce SOCS3 in hepatic cells [55], but nothing is known about the modulation of SOCS3 expression by the cytokine in human epidermal keratinocytes. For this purpose, mRNA expression of SOCS family members was firstly analyzed in IL-22-activated keratinocytes. We found that IL-22 rapidly induced high amounts of SOCS3 mRNA (Fig. 6.1A), whereas the expression of the other SOCS could not be affected by cytokine treatment. Next, we manipulated the SOCS3 expression by transient RNA interference in cultured keratinocytes and analyzed the IL-22-triggered STAT3 activation. SOCS3-silenced cells showed a significant increase of STAT3 Tyr705 phosphorylation after IL-22 treatment compared to cells transfected with irrelevant siRNA (Fig. 6.1B, left panels). Conversely, SOCS3 depletion did not affect the phosphorylation in Ser727 of STAT3. To better clarify the involvement SOCS molecules in the control of IL-22-induced STAT3 activation, the keratinocyte-like cell line HaCaT was stably transfected with plasmids encoding *c-myc*-tagged SOCS1, SOCS2, or SOCS3 molecules. Genetycin-resistant clones were screened for SOCS expression levels, and four SOCS1 clones, two SOCS2 clones and three SOCS3 clones were obtained and included in the study. SOCS3 keratinocyte clones strongly reduced the IL-22-mediated STAT3 phosphorylation in Tyr705 but not in Ser727 residue (Fig. 6.1B, right panels). Interestingly, even though SOCS1 was not up-regulated by IL-22, STAT3 Tyr705 phosphorylation was greatly inhibited in all keratinocyte clones expressing SOCS1, whereas no differences were observed for cells permanently transfected with SOCS2 (Fig. 6.1B, right panels). Keratinocyte clones expressed comparable levels of SOCS1, SOCS2, and SOCS3 proteins (Fig. 6.1B, right panels). In addition, transient transfections of keratinocytes with SOCS1 or SOCS3 plasmids markedly reduced the IL-22-induced transactivation of the STAT3-responsive plasmid pTKS3-Luc (Fig. 6.1C). In contrast, transient SOCS2 overexpression in keratinocytes had no effects on STAT3 activity.

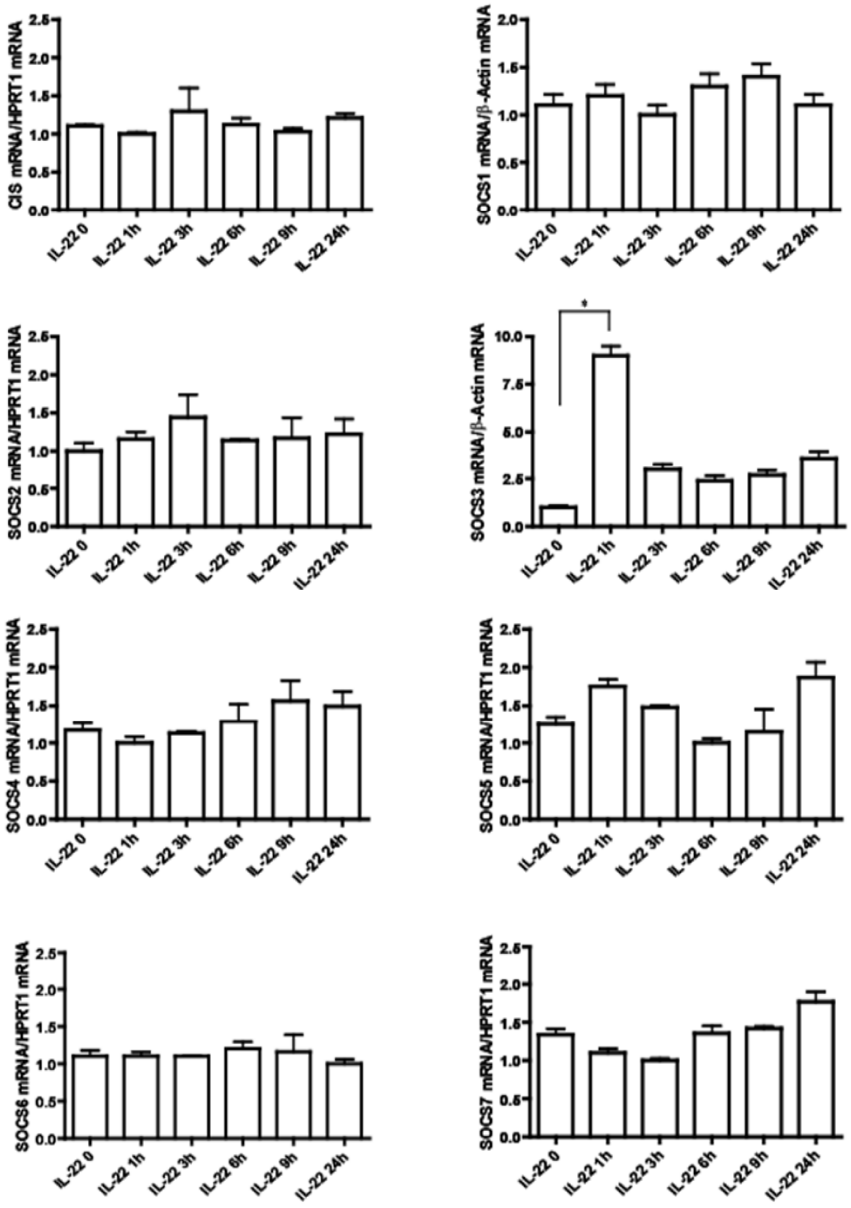

Figure 6.1A: SOCS3 is rapidly induced by IL-22 in keratinocytes. Real-time PCR analyses for CIS and SOCS1-7 were performed on reverse-transcribed RNA from primary culture of keratinocytes treated with IL-22 as indicated. **P* <0.05.

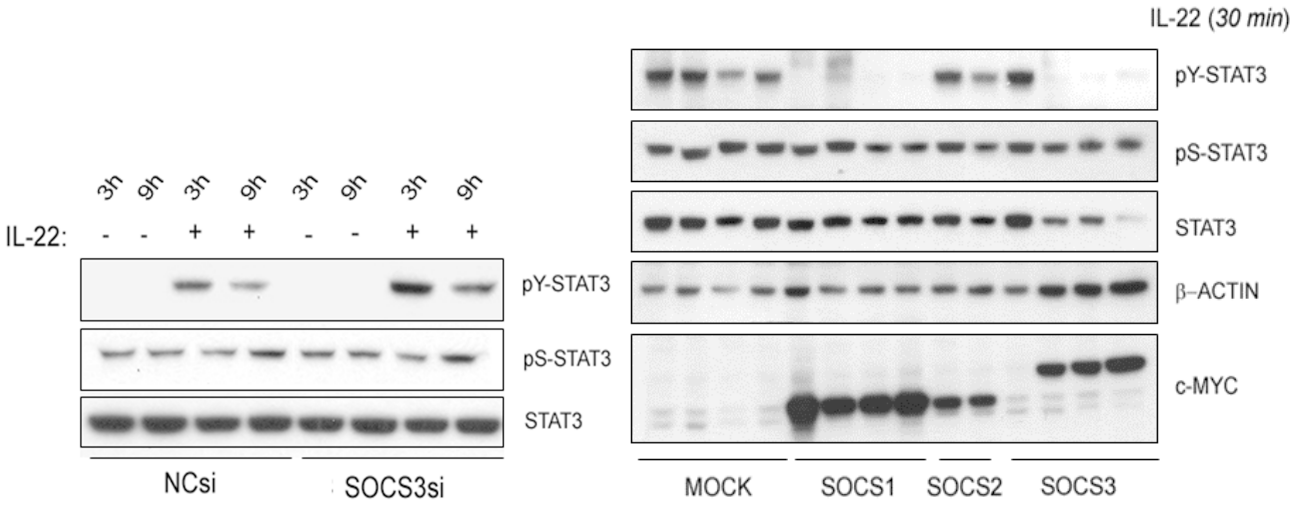

Figure 6.1B: SOCS3 inhibits the IL-22-induced STAT3 Tyr705 phosphorylation. Western blotting performed on lysates from SOCS3-silenced primary keratinocytes (left panels) or HaCaT cells stably transfected with SOCS1, SOCS2 or SOCS3 plasmids (right panels) activated or not with IL-22 for the indicated time.

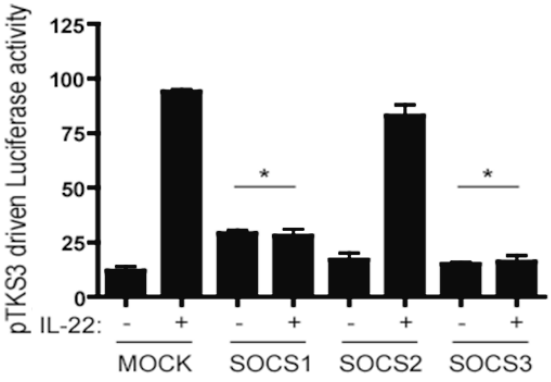

Figure 6.1C: IL-22-dependent STAT3 activity is counter-regulated by SOCS3. Representation of luciferase activity of keratinocytes transiently co-transfected with the pTKS3Luc and SOCS1, SOCS2, SOCS3 or empty (MOCK) plasmids. Cultures were treated or not with IL-22 for 8-h. 3 independent experiments were performed. Data are expressed as means ± SD of *Firefly* luciferase values normalized to *Renilla* luciferase and micrograms of total proteins. **P*<0.05.

6.2 SOCS3 inhibits the IL-22-induced inflammatory gene expression and proliferative effects in keratinocytes.

Since an inhibitory effect of SOCS3 on IL-22-dependent STAT3 activation was observed, the possible role of the molecule in regulating the expression of STAT3-dependent genes induced by IL-22 in keratinocytes was evaluated. As expected, SOCS3 mRNA silencing in IL-22-treated keratinocytes up-regulated the expression of CXCL1 and CXCL8 mRNA compared to control cells (Fig. 6.2A). In addition, HBD-2 gene expression, which was not STAT3-dependent, was also positively influenced by SOCS3 silencing (Fig. 6.2A). To clarify the role of SOCS3 in the control of IL-22-induced keratinocyte proliferation, Crystal violet tests were performed on cultured keratinocytes after the manipulation of SOCS3 expression. SOCS3-silenced keratinocytes proliferated more actively in resting condition as well as in response to IL-22 compared to cells transfected with irrelevant siRNA (Fig. 6.2B, left graph). *Vice versa*, SOCS3 overexpressing keratinocytes were quite totally resistant to the strong proliferative effect induced by IL-22 3-d of stimulation (Fig. 6.2B, right graph). Consistently with STAT3 inhibition data, SOCS1 clones did not respond to the proliferative effects of IL-22.

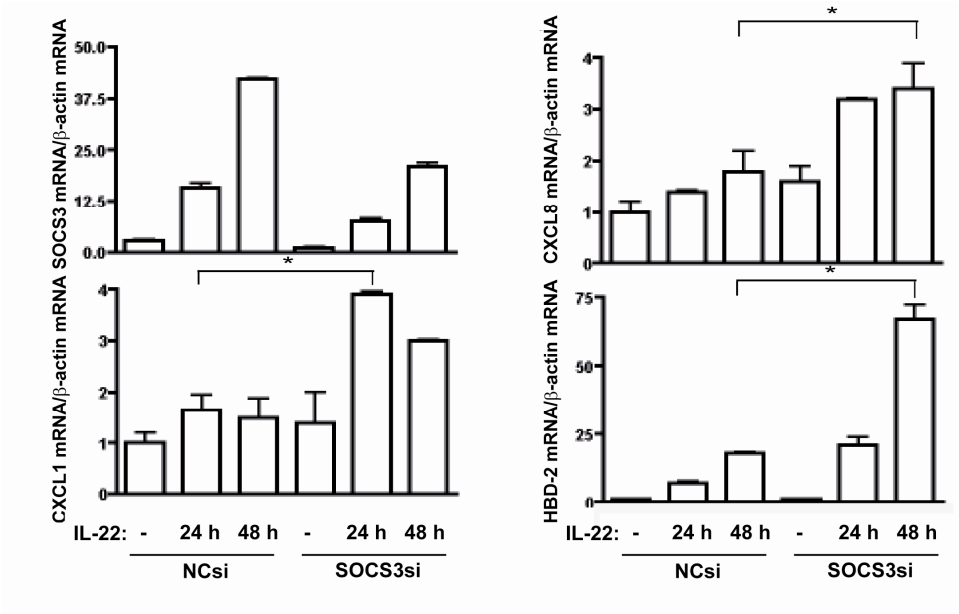

Figure 6.2A: The expression of IL-22-induced genes is reduced by SOCS3. SOCS3, CXCL8, CXCL1 and HBD-2 mRNA expression was analyzed in samples silenced for SOCS3 or treated with NC siRNA and then stimulated with IL-22 as indicated. **P*<0.05.

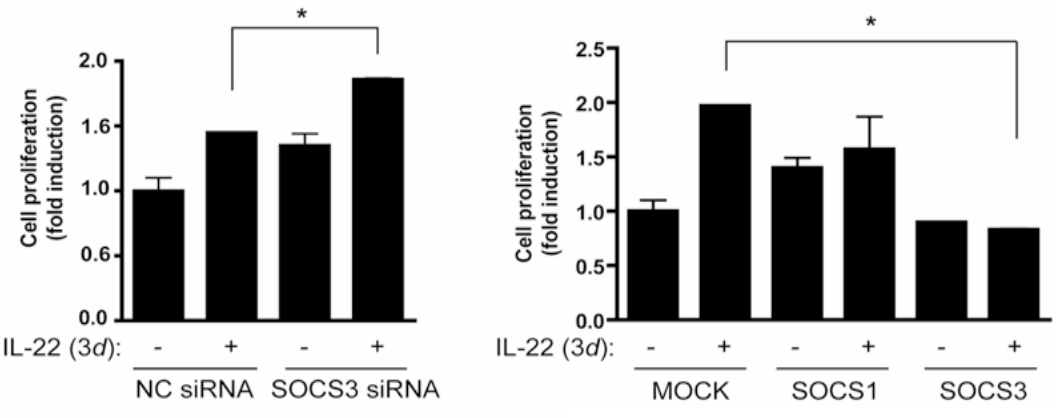

Figure 6.2B: SOCS3 inhibits the IL-22-mediated keratinocyte proliferation. Crystal violet assay was performed on keratinocytes transfected with SOCS3 or irrelevant siRNA and 3-d treated with IL-22 (left graph). Proliferation was also analyzed in SOCS3, SOCS1 or MOCK stably transfected cells kept in culture for 3 d in the presence or absence of IL-22. Proliferation was then analyzed by measuring at a spectrophotometer the incorporation of the crystal violet dye. Data are expressed as fold-induction of IL-22-treated over untreated samples, which were given a value of 1. *$P < 0.05$.

6.3 SOCS3 inhibitory effect on IL-22-induced STAT3 activation is mediated by SOCS3-KIR domain.

The last step was to identify SOCS3 regions responsible for the abrogation of the IL-22-triggered STAT3 activity executed by SOCS3. For this purpose, the pTKS3 luciferase activity was measured in cultured keratinocytes transiently transfected with plasmids encoding SOCS3 mutated in different domains, and then stimulated with IL-22 for 8 h. In particular, dN25 (KIR-deleted), dN36 (KIR- and part of ESS-deleted), L22D (single-mutation in KIR), R71E (single-mutation in SH2) and dC40 (SOCS box-deleted) plasmids were used in this study. We observed that a single amino acid mutation in SOCS3-KIR domain was sufficient to abrogate the inhibitory effect of the wild-type SOCS3 (WT) on the IL-22-induced pTKS3 luciferase activity (Fig. 6.3). In parallel, a significantly up-regulation in STAT3 transcriptional activity was found by trasfecting keratinocytes with dN25 and dN36 KIR-deleted plasmids. In addition, the expression of the SOCS3-SH2 mutant (R71E) in keratinocytes, which abrogated the SOCS3 capability to bind protein targets, also enhanced the luciferase activity of the

STAT3 responsive plasmid compared to cells expressing SOCS3 WT. Conversely, dC40 plasmid, in which SOCS-Box region was deleted, more efficiently inhibited the IL-22-mediated STAT3 luciferase activity compared to the SOCS3 WT. This was due to the fact that the SOCS-Box deletion impeded the proteasome-degradation of SOCS3, therefore enhancing its levels. All these data suggested that the SOCS3-KIR domain has a pivotal role in the SOCS3 inhibitory effect on IL-22 signaling in keratinocytes.

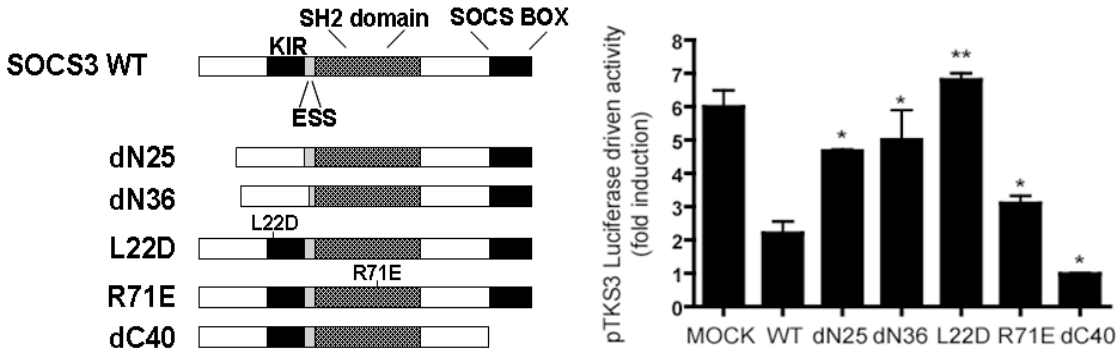

Figure 6.3: SOCS3 KIR domain is pivotal in the inhibition of the IL-22-dependent STAT3 transcriptional activity. Representation of luciferase activity of keratinocytes transiently co-transfected with the pTKS3Luc and SOCS3 WT, dN25, dN36, L22D, R71E, dC40 or empty plasmids. Cultures were treated or not with IL-22 for 8 hours. 3 independent experiments were performed. Data are expressed as means ± SD of *Firefly* luciferase values normalized to *Renilla* luciferase and micrograms of total proteins*$P < 0.05$; **$P < 0.01$.

7. Discussion

Psoriasis results from complex, aberrant relationships between the skin and immune system as well as genetic predisposition and environmental factors [2]. Epidermal keratinocytes of psoriatic skin show an exaggerated wound healing response and an aberrancy of STAT3 activation in keratinocytes has been proposed for the pathogenesis of psoriasis [61]. Indeed, STAT3 is activated in keratinocytes of psoriatic patients [31]. This up-regulation of STAT3 activation in psoriasis does not appear to be a secondary outcome of epidermal hyperplasia, because lesions from non-psoriatic inflammatory skin diseases with characteristic acanthosis show a STAT3 staining pattern similar to normal epidermis. In addition, increased levels of cytokines or growth factors that promote STAT3 activation have been found within psoriatic lesions [5, 61]. Among them, IL-22 potently triggers STAT3 activation and consequently alters the proliferative and differentiative processes in keratinocytes, and induces inflammatory molecules [24]. Therefore, the inhibition of IL-22-induced STAT3 activation in keratinocytes could be a valid strategy for the treatment of psoriasis. Besides phosphorylation on tyrosine 705 and serine 727 sites within the carboxyl-terminal region, STAT3 proper activation requires the acetylation on a single lysine residue 685, which is executed by histone acetyl transferase p300 [19, 20]. The acetylation of STAT3 was found to be critical for it to form stable dimers, which are required for cytokine-stimulated DNA binding and transcriptional regulation. STAT3 activation is negatively regulated through numerous mechanisms. These involve deacetylase enzymes, SOCS molecules, protein inhibitor of activated STAT (PIAS), protein phosphatases, and ubiquitination-dependent proteosomal degradation [62]. In this study we analyzed the role of SIRT1 deacetylase enzyme and SOCS3 molecule in the control of the IL-22-induced and STAT3-dependent effects in human keratinocytes. It is well known that SIRT1 can regulate the acetylation status and, thus, the phosphorylation and function of several transcription factors, including p53, E2F1, NF-κB, c-Jun, and members of the FoxO family [63]. Interestingly, Nie *et al*. [39] have described the involvement of SIRT1 in promoting STAT3 Lys685 deacetylation, and, thus, in down-regulating STAT3 phosphorylation and activity in hepatocytes during gluconeogenesis. STAT3 acetylation is rarely found in resting cells but it can be efficiently induced by cytokines, such as oncostatin M, IL-6, and by type I IFNs [35]. Conversely, we found that in keratinocytes STAT3 is constitutively acetylated in Lys685 residue, which is indispensable for the IL-22-triggered STAT3 activation. SIRT1 negatively regulated STAT3 acetylation, and consequently inhibited the IL-22-induced STAT3 phosphorylation in Tyr705.

In line with previous works suggesting that SOCS3 is a negative-feedback regulator of cytokine-induced STAT3 activation [51], we found that, other than SIRT1, also SOCS3 inhibited the IL-22-induced STAT3 Tyr705 phosphorylation in keratinocytes. SIRT1, PIAS proteins and protein tyrosine phosphatases target STAT3 directly, whereas SOCS3 through its KIR domain down-regulated the upstream kinase activity responsible for STAT3 phosphorylation. Therefore, it is reasonable to think that SOCS3 potentially could block some IL-22-activated signaling pathways other than STAT3. Moreover, both SIRT1 and SOCS3 inhibited IL-22-induced STAT3 transcriptional activity and as a direct consequence, reduced the STAT3-dependent expression of CXCL1 and CXCL8 chemokines. We also found that SIRT1 decreased the IL-22-induced and STAT3-dependent nuclear translocation of typical markers of keratinocyte proliferation (PCNA, cyclin D1, and pRB) as well as it increased the levels of markers of keratinocyte differentiation (KRT1 and loricrin). Since IL-22 exerts a mitogenic activity in keratinocyte cells [17], we then evaluated if SIRT1 and SOCS3 could also regulate the cytokine-induced biological effects in cultured keratinocytes. Through the manipulation of SIRT1 and SOCS3 expression, we found that these molecules opposed the IL-22-mediated proliferation of cultured keratinocytes. In addition, SIRT1 counteracted the cytokine-derived wound healing. Since the IL-22-induced closure of keratinocyte layer was strictly dependent on STAT3, it is possible that the negative effects of SIRT1 on IL-22-induced wound healing were explicated through the inhibition of STAT3 acetylation. SIRT1 inhibitory function on proliferative processes of keratinocytes is consistent with previous data demonstrating that the enzyme is an inhibitor of keratinocyte proliferation and an inducer of keratinocyte differentiation [42]. The anti-proliferative role of SOCS3 was in line with a previous work demonstrating that keratinocytes from transgenic mice over-expressing SOCS3 show a reduced migration and proliferation *in vitro* [64]. From the *in vivo* analysis of SIRT1 expression we found that it was mainly localized at the basal layer of the epidermis of both healthy and psoriatic skin, where keratinocytes have a high mitotic activity. In fact, SIRT1 drives proliferative keratinocytes to the differentiative programs necessary for the formation of the upper layers of the epidermis. However, compared to healthy and not lesional psoriatic skin, the expression of SIRT1 was reduced in the basal layer of lesional epidermis especially at the tips of the epidermal papillae close to the IFN-γ- producing $CD3^+$ T-cell infiltrate. IFN-γ reduced SIRT1 expression also *in vitro* and as a consequence it enhanced STAT3 Lys685 acetylation. Moreover, IFN-γ pretreatment of keratinocyte cultures strongly enhanced STAT3 phosphorylation in Tyr705 and STAT3-dependent gene expression induced by IL-22. The amplificatory effect of IFN-γ on IL-22 signaling in keratinocytes is analogous to that promoted by TNF-

α, even if the molecular mechanisms and genes regulated by the combination of IL-22 and TNF-α are completely different [11]. The specificity of IFN-γ and TNF-α effects on IL-22 signaling could depend on their ability to regulate and activate different molecular pathways. Concerning STAT3, TNF-α, differently from IFN-γ, did not affect its phosphorylation nor acetylation. Other than potentiating IL-22 signaling in keratinocytes, IFN-γ was also able to counteract IL-22-induced downstream effects in keratinocytes. In fact, it could reduce both basal and IL-22-promoted PCNA nuclear accumulation, accordingly to its potent anti-proliferative effect on this cell type. Therefore, IFN-γ can either potentiate or inhibit IL-22 signaling, with this dual effect depending on the IFN-γ ability to induce, in addition to STAT3 acetylation, other molecular cascades. Among these, STAT1 activation, usually associated with a decrease in proliferation rate in IFN-γ-sensitive cells, could be responsible for the dominant inhibitory effect of IFN-γ on IL-22-induced PCNA expression. It would be important to evaluate whether IFN-γ has a prevailing role also on IL-22-induced proliferation in keratinocytes, and whether psoriatic keratinocytes are less or more sensitive than healthy cells to the anti-proliferative or mitogenic effect of IFN-γ and IL-22, respectively. Indeed, previous studies have already shown that psoriatic keratinocytes aberrantly respond to IFN-γ, whose injection into uninvolved psoriatic skin causes epidermal hyperplasia and plaque development [65]. The enhanced proliferative response of psoriatic keratinocytes to IFN-γ has been supposed to rely on a reduced activation of STAT1 signaling and on an altered expression level of the IFN-γ receptor complex in the epidermis of psoriatic lesions compared to healthy skin [66].

Although, SOCS3 was highly expressed in the epidermis of psoriatic skin (data not shown), this was not sufficient to inhibit the detrimental effects triggered by IL-22 and other pathogenetic cytokines in keratinocytes. Importantly, we have demonstrated that SOCS3 not only exerts protective effects through the inhibition of STAT3 activation, but it was also able to sustain the IL-22-triggered activation of ERK1/2, which drives pro-survival programs in keratinocytes and could ultimately contribute to the expression of the peculiar epidermal thickening of psoriatic skin. The enhancement of the IL-22-induced ERK1/2 phosphorylation could be executed by SOCS3 similarly to the mechanism described by Madonna *et al*. for SOCS1 in IFN-γ-derived ERK1/2 activation [56]. We think that SOCS3, through its SOCS-Box domain, induced the degradation of Ras-Gap, the activation of Ras and finally the phosphorylation of ERK1/2.

Taken together these data suggest that the use of activators of SIRT1 or molecules able to specifically mimic the SOCS3-KIR region, capable to decrease STAT3 activation in epidermal keratinocytes, may

be therapeutically relevant for the treatment of psoriasis but also for other skin diseases characterized by aberrant STAT3 activation. The latter include cutaneous tumors, such as papilloma and squamous cell carcinoma, where STAT3 has a role in the tumor promotion stage of epithelial carcinogenesis [67]. Indeed, STAT3 inhibition by STAT3 decoy oligonucleotides in mice harboring activated STAT3 abrogated the *de novo* generation of tape stripping-induced psoriatic lesions in mice overexpressing STAT3 in the epidermis as well as the development of TPA-induced skin carcinoma [5, 61, 67]. In addition, the STAT3 inhibitor STA-21 molecule, an antibiotic which blocks STAT3 dimerization and DNA binding, has been successfully employed in a pilot study for the treatment of psoriasis [32].

8. References

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9. Acknowledgements

I would like to express my gratitude towards all those people who have helped me in completing my thesis and without whose support this work would be impossible. First of all, I would like to thank Prof. Giampiero Girolomoni, which has allowed me to be his PhD student at the Verona University. Therefore, I would like to thank Dr. Andrea Cavani for giving me the opportunity to carry out the research activity of my thesis in the Laboratory of Experimental Immunology at the Istituto Dermopatico dell'Immacolata (IDI)-IRCCS in Rome. I would particularly like to thank my supervisor Dr. Cristina Albanesi. Her ideas, guidance and suggestions made possible to realize this thesis. I also thank Dr. Claudia Scarponi and Dr. Stefania Madonna, which performed some key experiments of this thesis and never hesitated to provide help whenever I require. These last three persons importantly contributed to my professional and human growth and I'll never stop thanking them. Finally, I extend my thanks to Dr. Saveria Pastore for her many life and scientific tips, and all my colleagues for their assistance and collaboration during these three years.

10. Manuscripts

The FASEB Journal article fj.10-172288. Published online November 23, 2010.

STAT3-dependent effects of IL-22 in human keratinocytes are counterregulated by sirtuin 1 through a direct inhibition of STAT3 acetylation

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ABSTRACT **IL-22 has a pathogenetic role in psoriasis, where it is responsible for the altered proliferation and differentiation of keratinocytes and induces inflammatory molecules. The IL-22-induced effects are mediated by STAT3, whose activity is proportional to acetylation in lysine (Lys)685 and phosphorylation in tyrosine (Tyr)705. Lys 685 acetylation of STAT3 is inhibited by sirtuin (SIRT)1, a class III deacetylase promoting keratinocyte differentiation. Due to the opposite effects of IL-22 and SIRT1, we investigated whether IL-22-induced effects in keratinocytes could be regulated by SIRT1 through control of STAT3. We found that SIRT1 opposes the IL-22-induced STAT3 activity by deacetylating STAT3 and reducing STAT3 Tyr705 phosphorylation. By controlling STAT3, SIRT1 also influences the IL-22-induced expression of molecules involved in proliferation and inflammation as well as proliferation and migration processes in cultured keratinocytes. Although SIRT1 levels were similar in keratinocytes of healthy individuals and patients with psoriasis, they were reduced in psoriatic skin lesions, with the lymphokine IFN-γ inhibiting SIRT1 expression. Concomitantly, IFN-γ enhanced basal acetylation of STAT3 and its phosphorylation induced by IL-22. In conclusion, STAT3-dependent IL-22 signaling and effects in keratinocytes are negatively regulated by SIRT1. In skin affected by psoriasis, SIRT1 is downregulated by IFN-γ, which thus renders psoriatic keratinocytes more prone to respond to IL-22.—Sestito, R., Madonna, S., Scarponi, C., Cianfarani, F., Failla, C. M., Cavani, A., Girolomoni, G., Albanesi, C. STAT3-dependent effects of IL-22 in human keratinocytes are counterregulated by sirtuin 1 through a direct inhibition of STAT3 acetylation. *FASEB J.* 25, 000–000 (2011). www.fasebj.org**

Key Words: class III histone deacetylase (HDAC) · post-translational modification · psoriasis · skin inflammation · IFN-γ

IL-22 IS A MEMBER OF THE IL-10 family preferentially produced by a subset of T-helper (Th)1 and Th17 cells (1, 2), and by the recently identified Th22 subset (3–5). IL-22 acts through a heterodimeric receptor containing the IL-10 receptor (R)2 and IL-22R1 chains (6). IL-10R2 is widely expressed on immune cells, whereas IL-22R1 expression is restricted to nonhematopoietic cells, such as epidermal keratinocytes and epithelial cells of the gastrointestinal tract and the lung (7, 8). IL-22 has been considered as a key cytokine in psoriasis, an immune-mediated skin disease characterized by a marked epidermal hyperplasia associated with altered keratinocyte differentiation (9–11). In psoriasis, IL-22 mediates keratinocyte proliferation, inhibits keratinocyte terminal differentiation, and stimulates the production of antimicrobial peptides and chemokines (9–11). STAT3 is the principal mediator of IL-22 signaling (9); its levels are increased in psoriatic epidermis, and overexpression of STAT3 in keratinocytes determines a psoriatic phenotype in transgenic mice (12). STAT3 activation requires STAT3 phosphorylation in Tyr705 and in Ser727 residues (13), and can be counterregulated by the suppressor of cytokine signaling (SOCS)3 in a classic feedback loop (14). Notably, STAT3 activation is proportional to acetylation in Lys685 residue, since this post-translational modification is required for phosphorylation in Tyr705 (15). Acetylation of STAT3 relies on a tight balance between acetylation and deacetylation performed primarily by p300 acetylase and histone deacetylase (HDAC) enzymes, respectively (15). In particular, SIRT1, the best characterized member of class III HDACs, mediates deacetylation of the STAT3 Lys685 site and, in turn, negatively regulates STAT3 phosphorylation in Tyr705 (16). Recently, it has been shown that SIRT1 is an important inducer of keratinocyte differentiation and inhibitor of keratinocyte proliferation (17). These SIRT1-mediated effects are specific of keratinocytes and are not shared by other cell types, such as white adipocytes and myocytes, where

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doi: 10.1096/fj.10-172288

SIRT1 overexpression counteracts differentiative processes (18, 19).

Due to the opposite effects of IL-22 and SIRT1 in keratinocytes, we investigated whether IL-22-induced molecular signaling, gene expression, and proliferation could be regulated by SIRT1 through direct control of STAT3 acetylation and phosphorylation. Specifically, we analyzed the effects of SIRT1 on IL-22-regulated expression of genes involved in proliferation, innate immunity, and inflammation, and genes typical of initial and terminal epidermal differentiation, with a particular interest in STAT3-dependent genes. We found that SIRT1 opposed STAT3 activation and STAT3-dependent effects of IL-22 on keratinocytes, by deacetylating STAT3 and reducing STAT3 phosphorylation in Tyr705. Although SIRT1 levels were similar in cultured keratinocytes isolated from skin of healthy individuals and patients with psoriasis, they were reduced *in vivo* in the epidermal papillae of psoriatic lesions, especially in areas closer to the inflammatory $CD3^+$ T-cell infiltrate. It is worth noting that IFN-γ, mostly released by Th1 cells infiltrating psoriatic skin, inhibited SIRT1 expression and, concomitantly, enhanced STAT3 Lys685 acetylation and phosphorylation in Tyr705, rendering keratinocytes more prone to express IL-22-inducible genes.

MATERIALS AND METHODS

Tissue samples

For immunohistochemistry, 4-mm punch biopsies were taken from lesional (LS) skin of adult patients with chronic plaque psoriasis (n=6) and from normal skin of healthy subjects undergoing plastic surgery (n=4). For patients with psoriasis, a skin biopsy from nonlesional (NLS) skin distant from the plaques was also taken. Keratinocyte cultures were established from 6-mm biopsies obtained from both involved and uninvolved skin of patients with psoriasis (n=6) and from healthy control subjects (n=8). T-cell clones were established from the peripheral blood of 3 patients with psoriasis. The study was approved by the Ethical Committee of the IDI-IRCCS (Rome, Italy).

Immunohistochemistry

Cryostatic sections were incubated with monoclonal antibodies (mAbs) against SIRT1 (E104; 1:100 dilution; Abcam, Cambridge, UK) or CD3 (1:20 dilution; BD-Pharmingen, Franklin Lakes, NJ, USA). Secondary biotinylated mAbs and staining kits (Vector Laboratories, Burlingame, CA, USA) were used to develop immunoreactivity. Figures depict one experiment that is representative of all the patients investigated.

Keratinocyte cultures and treatments

Primary cultures of human keratinocytes were obtained from skin biopsies, as described previously (20). Keratinocyte cultures were grown in serum-free keratinocyte growth medium (KGM; Clonetics, Walkersville, MD, USA), for ≥3–5 d (at 60–80% confluence) before performing experiments. Stimulations with 50 ng/ml IL-22, 200 U/ml IFN-γ, 50 ng/ml TNF-α, or 50 ng/ml IL-17 (all from R&D Systems, Minneapolis, MN, USA) were performed in keratinocyte basal medium (KBM, Clonetics). Subconfluent cultures were also stimulated with supernatants from Th1 clones or RPMI medium diluted 1:3 in KBM. Terminal differentiation of keratinocyte cultures was achieved by growing cells at 100% of confluence (t_0) and, thus, keeping them in culture for another 4 d. Keratinocytes undergoing differentiation were also cultured in the presence of IL-22 and/or IFN-γ.

Preparation of supernatants from T-cell clones obtained from patients with psoriasis

$CD4^+$ T-cell clones were obtained from peripheral blood of patients with psoriasis, as described previously (21). Cells were periodically stimulated with 1% phytohemagglutinin (Invitrogen, Carlsbad, CA, USA), and were activated with plate-coated anti-CD3 and soluble anti-CD28 (both at 1 μg/ml). Clone supernatants were assayed for IFN-γ, TNF-α, IL-17, IL-22, and IL-4 content with a commercially available ELISA kit (R&D Systems). Clones were classified depending on their cytokine profile, and supernatants of Th1 clones containing levels of IFN-γ > 20 ng/ml were used on keratinocyte cultures.

RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen), and analyzed by real-time PCR. The expression of CXCL8, CXCL1, SOCS3, SOCS1, S100A7, HBD-2, STAT3, STAT1, SIRT1, and β-actin mRNA were evaluated in the ABI Prism SDS 7000 PCR instrument (Applied Biosystems, Branchburg, NJ, USA), using SYBR Green PCR reagents or Taqman PCR Master Mix. The forward and reverse primers employed for real-time PCR were as follows: for CXCL8, 5′-GCTGGCTTATCTTCACCATCATG-3′ and 5′-TTATTTTTTTTCAGTTAATTAACAGATGCTATCAT-3′; for SOCS3, 5′-AAGGACGGAGACTTCGATTCG-3′ and 5′-AAACTTGCTGTGGGTGACCAT-3′; for SOCS1, 5′-TTTTTCGCCCTTAGCGTGA-3′ and 5′-AGCAGCTCGAAGAGGCAGTC-3′; for STAT3, 5′-GGCGTCACTTTCACTTGGGT-3′ and 5′-CCACGGACTGGATCTGGGT-3′; for STAT1, 5′-TTGCTTGGATCAGCTGCAGA-3′ and 5′-GCTGCAGACTCTCCGCAACTA-3′; for SIRT1, 5′-GCTGGCCTAATAGAGTGGCAA-3′ and 5′-CTCAGCGCCATGGAAAATG-3′, for β-actin, 5′-CATCGAGCACGGCATCGTCA-3′ and 5′-TAGCACAGCCTGGATAGCAAC-3′. The sequences of the primers and internal probe for HBD-2 mRNA have been previously described (22). Primers for CXCL1 and S100A7 were provided by Applied Biosystems (Hs00236937 and Hs00161488, respectively). The levels of gene expression were determined by normalizing to β-actin mRNA expression. The values obtained from triplicate experiments were averaged, and data are presented as means ± SD.

Flow cytometry analysis

Keratinocyte expression of membrane intercellular adhesion molecule (ICAM)-1, major histocompatibility complex (MHC) class I and II was evaluated using FITC-conjugated anti-CD54 (84H10; Immunotech, Marseille, France), anti-HLA-ABC (G46-2.6; BD-Pharmingen) and anti-HLA-DR (L243; BD-Pharmingen) mAbs. Cells were analyzed with a FACScan equipped with Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

Western blot analysis

Total proteins and cytosolic and nuclear extracts were prepared as previously reported (20), and subjected to SDS-PAGE. Western blotting filters were developed using the ECL-plus detection system (Amersham, Dübendorf, Switzerland) or the SuperSignal West Femto kit (Pierce, Rockford, IL, USA). The Abs employed for the study were as follows: anti-PCNA (PC10), anti-cyclin D1 (DCS-6), anti-phospho-RB (Ser795), anti-lamin A/C (346), anti-α-tubulin (B-7), anti-β-actin (C-11), anti-phospho-STAT3 (Ser727), anti-STAT3 (C-20), anti-phospho-STAT1 (Tyr701), anti-STAT1 (E-23), anti-phospho-ERK1/2 (E-4), anti-ERK1/2 (C16), and anti-SIRT1 (H-300), all provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-KRT1, anti-KRT5, anti-KRT14, and anti-loricrin Abs were from Covance (Emeryville, CA, USA), whereas anti-phospho-STAT3 (Tyr705) and anti-acetyl-STAT3 (K685) were from Cell Signaling Technology (Danvers, MA, USA). Finally, anti-FLAG (M2) and anti-SIRT7 (RB1973) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and Abgent (San Diego, CA, USA), respectively.

Transient RNA interference, transfections, and luciferase assay

STAT3, SIRT1, and SIRT7 were knocked down by using a pool of 4 small short interfering (si)RNAs (ON-TARGET*plus* SMART-pool, Dharmacon RNA Technology, Lafayette, CO, USA). In parallel, a pool of 4 nontargeting siRNAs was used as negative control. Primary cultures were transfected with STAT3, SIRT1, SIRT7, or irrelevant siRNA using Interferin reagent (Polyplus Transfection, New York, NY, USA). After 2 d of mRNA silencing, keratinocytes were treated with different stimuli. Cultured keratinocytes grown in 12-well plates were transiently transfected with the STAT3-responsive plasmid pLucTKS3 (a generous gift of Prof. J. Turkson, University of Central Florida, Orlando, FL, USA) by using Lipofectin reagent (Invitrogen). After transfection, cells were stimulated with IL-22, and *Firefly* luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). pRL-null plasmid encoding the *Renilla* luciferase was included in each transfection. For dose-response experiments with pcDNA3-hSIRT1-FLAG (kindly provided by Prof. F. Ishikawa, Kyoto University, Kyoto, Japan), cells were transfected with pLucTKS3 plasmid and pRL-null vector, using the pcDNA3.1 plasmid as negative control. STAT3-driven luciferase activity of pLucTKS3 plasmid was also assayed in cultured keratinocytes transfected with SIRT1 siRNA.

Crystal violet assay

Cells (2×10^4) were seeded in 96-well plates, and, the day after, starved in KBM. Culture stimulation with IL-22 was conducted either in the presence or absence of 10 μM Ex-527 or 40 μM resveratrol (both from Sigma-Aldrich). SIRT1-silenced cells were also used in some experiments. After 2–4 d of treatment, cells were stained with 0.5% crystal violet, whose incorporation was measured at 540 nm in an ELISA reader (model 3550 UV ELISA reader; Bio-Rad, Hercules, CA, USA).

Scratch wound healing assay

Keratinocytes were STAT3 or SIRT1 silenced, grown at 100% of confluence, and then scratched with the tip of a p-200 pipette to

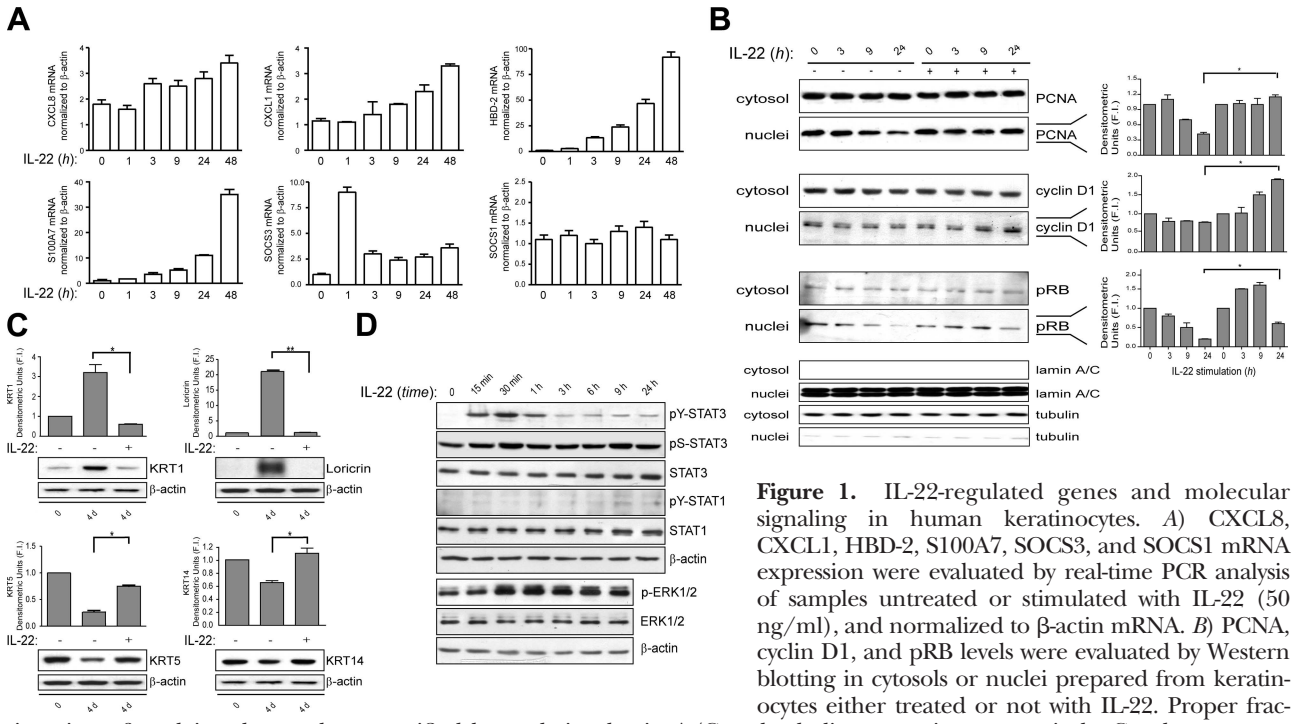

Figure 1. IL-22-regulated genes and molecular signaling in human keratinocytes. *A*) CXCL8, CXCL1, HBD-2, S100A7, SOCS3, and SOCS1 mRNA expression were evaluated by real-time PCR analysis of samples untreated or stimulated with IL-22 (50 ng/ml), and normalized to β-actin mRNA. *B*) PCNA, cyclin D1, and pRB levels were evaluated by Western blotting in cytosols or nuclei prepared from keratinocytes either treated or not with IL-22. Proper fractionation of nuclei and cytosols was verified by analyzing lamin A/C and tubulin expression, respectively. Graphs represent densitometric analyses of nuclear PCNA, cyclin D1, and pRB. Data are expressed as mean ± SD fold induction (F.I.), calculated relative to the untreated samples (t_0), which were given a value of 1. *C*) Keratinocyte cultures were grown at 100% of confluence (t_0) and left in culture for another 4 d in presence or absence of IL-22. Total lysates were analyzed by Western blotting using anti-KRT1, anti-KRT5, anti-KRT14, or anti-loricrin Abs. Graphs represent densitometric analyses of total KRT1, KRT5, KRT14, and loricrin normalized to β-actin signals. *D*) Total STAT3, STAT3 phosphorylated in Tyr705 and in Ser727, total STAT1, STAT1 phosphorylated in Tyr701, total ERK1/2, and ERK1/2 phosphorylated in Tyr204 were detected by Western blotting. Immunoblot stainings are representative of 3 independent experiments performed on 3 different keratinocyte strains. $^*P < 0.05$; $^{**}P < 0.01$.

create a uniform cell-free zone. Wounded monolayers were either incubated or not with 30 ng/ml IL-22. Microscopy pictures were taken with a digital camera at different time points following IL-22 treatment. The residual gap between migrating keratinocytes was measured with a computer-assisted image analysis system (Axiovision; Zeiss, Oberkochen, Germany), and expressed as percentage of the initial scratched area.

Densitometry and statistical analysis

Phospho-STAT3, acetyl-STAT3, PCNA, cyclin D1, pRB, KRT1, KRT5, KRT14, loricrin, and phospho-ERK1/2 immunoblots were subjected to densitometry using an imaging densitometer (GS-670; Bio-Rad). The significance of differences between densitometric values was determined by Wilcoxon's signed rank test (SigmaStat; Jandel, San Rafael, CA, USA). This test was also used to calculate the significance of differences between keratinocytes transfected with irrelevant and SIRT1-specific siRNAs as well as between cultures transfected with SIRT1-encoding and control plasmids in luciferase assays. Comparison of CXCL8, CXCL1, S100A7, HBD-2, and SOCS3 expression between keratinocytes transfected with irrelevant and SIRT1- or STAT3-specific siRNA, and of SIRT1 mRNA levels between untreated and cytokine-stimulated cultured cells was performed. Significant differences were also calculated for scratched cultures of keratinocytes. Values of $P \leq 0.05$ were considered significant.

RESULTS

Main mediator of IL-22-induced effects in human keratinocytes is STAT3

IL-22 controls several biological processes in keratinocytes, including proliferation, differentiation, migration, and production of molecules involved in the innate immune responses (23, 24). Our study of gene

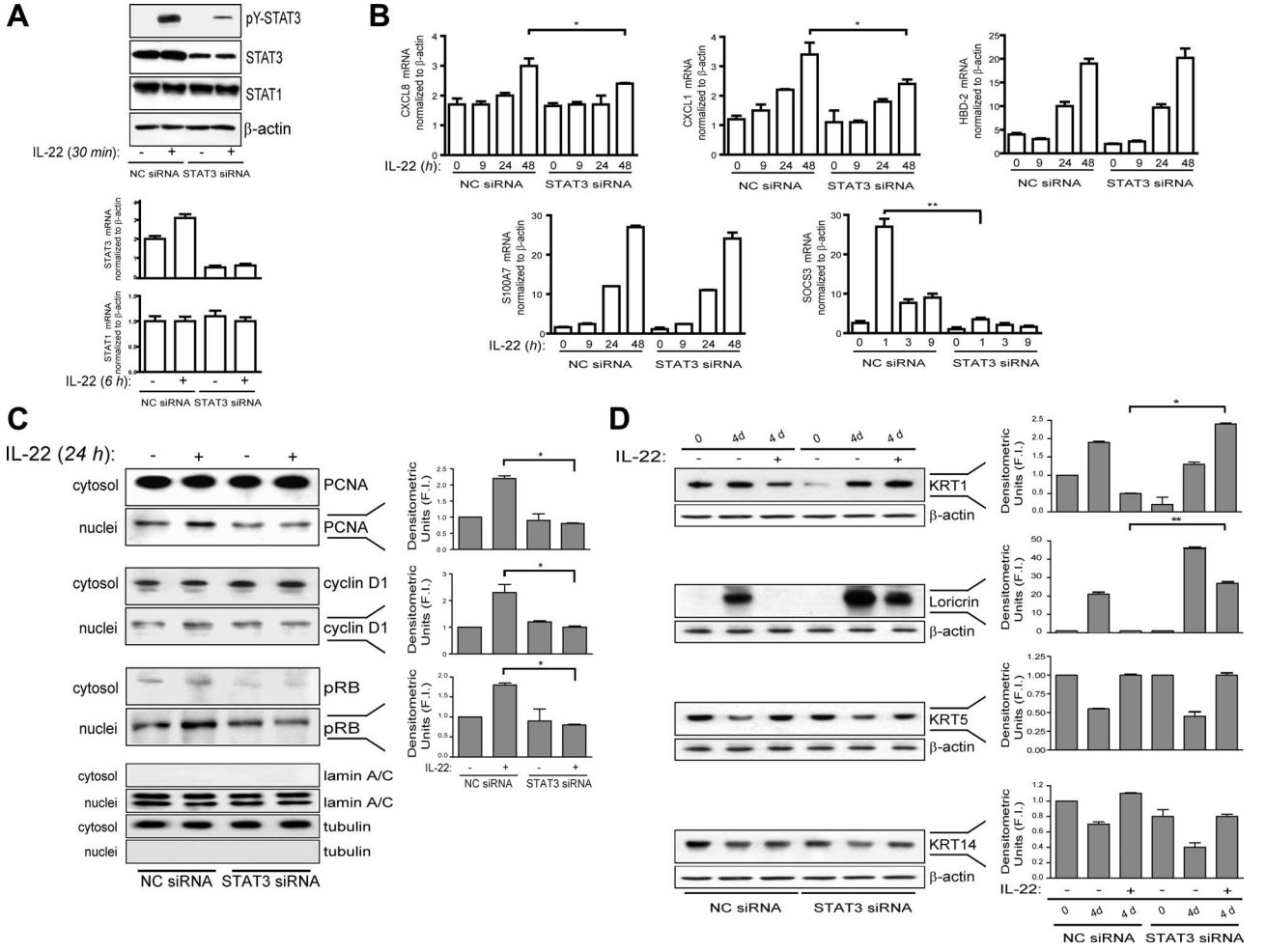

Figure 2. Majority of IL-22-regulated molecules in keratinocytes are STAT3-dependent. *A*) Keratinocyte cultures were transfected with siRNA specific for STAT3 or irrelevant siRNA (NC). After 48 h transfection, cells were stimulated with IL-22 or not for the indicated time periods. Analyses of STAT3 and STAT1 protein accumulation or mRNA expression were performed by Western blotting or real-time PCR, respectively. *B*) CXCL8, CXCL1, HBD-2, S100A7, and SOCS3 mRNA expression were analyzed in samples silenced for STAT3 or treated with NC siRNA. *C*) Western blotting of nuclei and cytosols served to study PCNA, cyclin D1, and pRB protein accumulation in untreated or IL-22-stimulated keratinocytes transfected with irrelevant and STAT3-specific siRNA. Graphs represent densitometric analyses of nuclear PCNA, cyclin D1, and pRB. *D*) STAT3-silenced keratinocyte cultures undergoing terminal differentiation (4 d) either activated or not with IL-22 were studied for KRT1, KRT5, KRT14, and loricrin expression by Western blotting. Graphs show densitometric values of KRT1, KRT5, KRT14, and loricrin. Western blots are representative of 3 independent experiments performed on 3 different keratinocyte strains. $*P < 0.05$; $**P < 0.01$.

and protein expression regulated by IL-22 in cultured human keratinocytes extended previous analyses (23, 24) and showed that IL-22 was able to up-regulate mRNA expression of CXCL8, CXCL1, HBD-2, and S100A7, with significant levels of induction at late time points of stimulation (24–48 h; **Fig. 1*A***). IL-22 also induced high amounts of SOCS3, which was rapidly induced (1 h), but did not affect SOCS1 mRNA expression. In parallel, we found that IL-22 could not regulate the membrane expression of ICAM-1, MHC class I and II molecules, induced by IFN-γ and TNF-α (data not shown). Although IL-22 is considered a potent inducer of epidermal hyperplasia in pathological conditions, little information exists about its effect on the induction of molecules controlling keratinocyte proliferation. To this end, IL-22-treated keratinocytes were analyzed for the expression of proteins inducing cell cycle progression and, thus, essential for cell proliferation (25). As shown in Fig. 1*B*, IL-22 maintained high nuclear levels of PCNA, cyclin D1 and pRB after 9 and 24 h, as compared to untreated cultures, which showed a decreased expression at these time points. In parallel, IL-22 could block the down-regulation of KRT5 and KRT14, two cytokeratins typical of proliferating keratinocytes, in cultures undergoing terminal differentiation (4 d of growth after confluence; Fig. 1*C*). *Vice versa*, IL-22 inhibited the expression of KRT1, a structural protein highly present in keratinocytes committed to terminal differentiation (Fig. 1*C*). IL-22 also potently decreased the protein accumulation of loricrin, which is a component of the cross-linked cell envelope in the uppermost layers of the epidermis (Fig. 1*C*). The analysis of IL-22-induced molecular cascades demonstrated that STAT3 signaling was rapidly induced in primary cultures of keratinocytes, and, in particular, STAT3 phosphorylation in Tyr705 was observed after 15–30 min of treatment with IL-22 (Fig. 1*D*). STAT3 phosphorylation in Ser727 residue, constitutively detected in keratinocytes, was also enhanced by IL-22, showing a peculiar biphasic kinetics (Fig. 1*D*). IL-22 did not affect STAT1 activation but significantly induced extracellular signal-regulated kinase (ERK)1/2 phosphorylation after 30 min of treatment (Fig. 1*D*).

To determine whether STAT3 expression and activation influenced the IL-22-induced regulation of CXCL8, CXCL1, HBD-2, S100A7, SOCS3, and of the proteins associated to keratinocyte proliferation and differentiation, STAT3 mRNA silencing was performed in IL-22-activated or untreated keratinocyte cultures (**Fig. 2*A***). Following silencing of STAT3 mRNA, keratinocytes were not able to up-regulate SOCS3 mRNA in response to IL-22 treatment, but continued to express HBD-2 and S100A7 (Fig. 2*B*). In parallel, CXCL8 and CXCL1 were only slightly induced by IL-22, indicating that STAT3 positively regulates these genes, but other mechanisms can control their expression. In addition, considering the delayed kinetics of CXCL8 and CXCL1 induction by IL-22, it is plausible that the STAT3 effect on these genes was indirect. Also, the nuclear accumulation of PCNA, cyclin D1, and pRB proteins observed after 24 h of incubation with IL-22 was significantly dependent on STAT3 expression (Fig. 2*C*). The negative effect of IL-22 on KRT1 expression did not occur when cells were STAT3 depleted (Fig. 2*D*). Similarly to KRT1, loricrin expression substantially increased in keratinocytes silenced for STAT3 (Fig. 2*D*), suggesting that STAT3 acts as a repressor of these molecules. On the contrary, the modulation of KRT5 and KRT14 expression by IL-22 treatment was independent from STAT3 (Fig. 2*D*). STAT3 mRNA and protein silencing by siRNA in keratinocytes was substantial

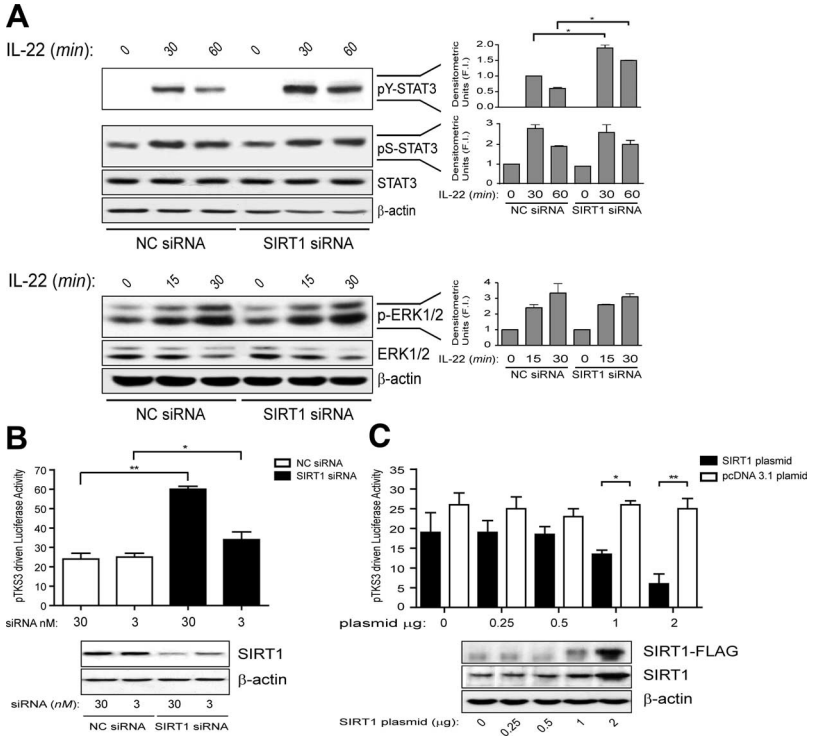

Figure 3. IL-22-induced STAT3 phosphorylation in Tyr705 and activity is counterregulated by SIRT1. *A*) Keratinocyte cultures were transfected with SIRT1-specific or irrelevant siRNA. After 48 h transfection, cells were stimulated with IL-22 or not, as indicated. Analyses of STAT3 phosphorylated in Tyr705 (pY-STAT3) and in Ser727 (pS-STAT3), total STAT3, total ERK1/2, and ERK1/2 phosphorylated in Tyr204 were performed by Western blotting. Densitometries of blots relative to pY-STAT3, pS-STAT3, and phosphorylated ERK1/2 are shown. *B*) Keratinocytes were treated with irrelevant siRNA or with siRNA specific for SIRT1, at 3 or 30 nM concentrations. Cells were then transiently transfected with pTKS3-Luc reporter plasmid and stimulated with IL-22. Effects of SIRT1 depletion on the STAT3-responsive plasmid were evaluated by assaying luciferase activity. *C*) Cultured keratinocytes were cotransfected with increasing amounts (0–2 μg/well) of pcDNA3.1 (open bars) or pcDNA3-hSIRT1-FLAG (solid bars), and of 0.5 μg pTKS3-Luc reporter plasmid; 3 independent experiments. Data are expressed as means ± SD of *Firefly* luciferase values normalized to *Renilla* luciferase and micrograms of total proteins. $*P < 0.05$; $**P < 0.01$.

and specific since STAT1 expression was unchanged (Fig. 2*A*). In summary, these data indicated that the majority of the IL-22-induced effects in human keratinocytes were mediated by STAT3 signaling.

SIRT1 opposes STAT3 activation and STAT3-dependent gene expression regulated by IL-22 in keratinocytes through the inhibition of STAT3 acetylation in Lys685

STAT3 is tightly regulated at the post-translational level, and, in particular, its acetylation in Lys685 is indispensable for the phosphorylation in Tyr705 and trans-activating function (15). Lys 685 acetylation of STAT3 can be, in turn, inhibited by SIRT1, which is also known to reduce proliferation and promote terminal differentiation of epidermal keratinocytes (16, 17). Therefore, we hypothesized that SIRT1 could oppose IL-22 signaling and effects on keratinocytes by inhibiting STAT3 acetylation in Lys685 and, thus, STAT3 phosphorylation and function. To this end, we manipulated SIRT1 expression by RNA silencing and analyzed STAT3 phosphorylation and activation after IL-22 treatment. SIRT1-silenced cells showed a significant up-regulation of STAT3 phosphorylation in Tyr705 residue compared to cells transfected with irrelevant siRNA (**Fig. 3*A***). In contrast, SIRT1 depletion did not affect the phosphorylation in Ser727 of STAT3 nor the activation of STAT1 and ERK1/2 (Fig. 3*A*). In addition, the analysis of STAT3 activity in IL-22-treated SIRT1-silenced keratinocytes transfected with a STAT3-responsive plasmid, pLucTKS3, revealed that SIRT1 negatively regulates STAT3 activity (Fig. 3*B*). On the contrary, SIRT1 overexpression by transfection with a human SIRT1-endoding plasmid dose-dependently inhibited the pTKS3 luciferase activity (Fig. 3*C*). These

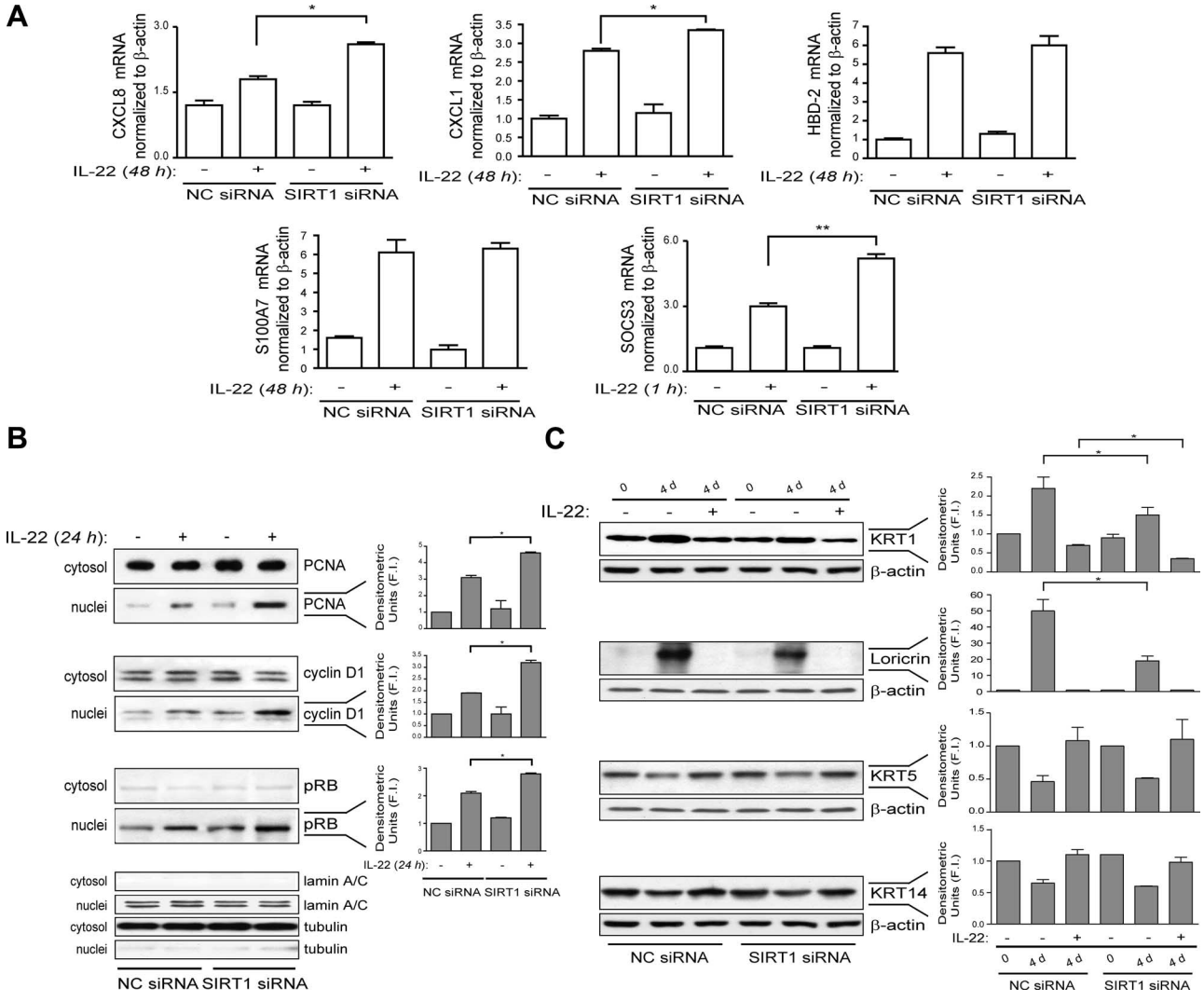

Figure 4. SIRT1 counteracts IL-22-regulated STAT3-dependent gene expression in keratinocytes. Keratinocyte cultures were transfected with SIRT1-specific or irrelevant siRNA, and then stimulated with IL-22 or not, as indicated. *A*) CXCL8, CXCL1, HBD-2, S100A7, and SOCS3 were analyzed by real-time PCR. *B*) PCNA, cyclin D1, and pRB were analyzed by Western blotting. *C*) KRT1, KRT5, KRT14, and loricrin proteins were also analyzed in keratinocytes committed to terminal differentiation. Graphs show densitometric values of PCNA, cyclin D1, pRB, KRT1, KRT5, KRT14, and loricrin staining on blots. Western blots are representative of 3 independent experiments performed on 3 different keratinocyte strains. $*P < 0.05$; $**P < 0.01$.

results led us to investigate the possible role of SIRT1 in regulating the expression of STAT3-dependent genes induced by IL-22 in keratinocytes. SIRT1 silencing in IL-22-treated keratinocytes strongly up-regulated SOCS3 mRNA expression (**Fig. 4*A***). CXCL8 and CXCL1 expression also increased in SIRT1-depleted keratinocytes compared to control cells (Fig. 4*A*). As expected, HBD-2 and S100A7 gene expression, which were not STAT3-dependent, were not influenced by SIRT1 silencing (Fig. 4*A*). Consistently, SIRT1-depletion enhanced the positive effect of IL-22 on PCNA, cyclin D1 and pRB nuclear translocation, which occurred in a STAT3-dependent manner (Fig. 4*B*). SIRT1-silencing also determined a marked reduction of KRT1 and loricrin expression in keratinocytes undergoing differentiation, in agreement with previous findings (17) (Fig. 4*C*). Of note, SIRT1 depletion reinforced the inhibitory effect of IL-22 on KRT1 expression (Fig. 4*C*). Finally, KRT5 and KRT14 up-regulation by IL-22 in keratinocytes undergoing differentiation *in vitro*, an event found to be independent from STAT3 (Fig. 2*D*), was not affected by SIRT1 silencing (Fig. 4*C*).

The finding that SIRT1 opposes IL-22 effects by counteracting STAT3 phosphorylation in Tyr705 led us to investigate the capability of SIRT1 to modulate STAT3 acetylation. We first analyzed STAT3 acetylation in Lys685 by Western blotting in keratinocytes untreated or activated with IL-22. STAT3 acetylation was constitutive and not modulated by IL-22 (**Fig. 5*A***). When keratinocytes were depleted of SIRT1, Lys685 constitutive acetylation of STAT3 was significantly enhanced compared to control cells, as assessed using different keratinocyte strains (n=3) and in independent experiments of silencing (3 representative experiments are shown in Fig. 5*B*). The effect of SIRT1 was specific since SIRT7, another sirtuin family member, did not influence STAT3 acetylation in Lys685 (Fig. 5*B*). Collectively, these data demonstrated that SIRT1 negatively modulates STAT3 capability to control the expression of molecules regulated by IL-22 by inhibiting Lys685 acetylation of STAT3 and, thus, its phosphorylation in Tyr705.

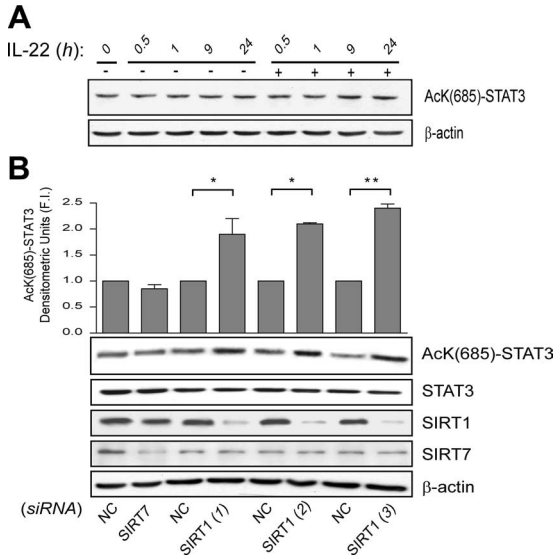

Figure 5. SIRT1 opposes STAT3 acetylation in Lys685. *A*) STAT3 acetylation in Lys685 was analyzed by Western blotting performed on untreated or IL-22-activated keratinocytes. Blots are representative of 3 independent experiments. *B*) STAT3 acetylation was also studied in keratinocytes transfected with siRNAs specific for SIRT1 or SIRT7, and compared to NC. Results are representative of 6 independent experiments.

IL-22-induced keratinocyte proliferation and migration is counteracted by SIRT1

To further clarify the role of SIRT1 in the control of IL-22-induced keratinocyte proliferation, we manipulated SIRT1 function and expression by using chemical modulators of SIRT1 or through RNA interference, respectively, and performed functional *in vitro* assays measuring the keratinocyte proliferative rate. Crystal violet tests on keratinocyte cultures treated with Ex-527, a specific inhibitor of SIRT1 enzymatic activity, or with resveratrol, a polyphenol stimulating SIRT1 deacetylase function, showed that SIRT1 significantly opposed IL-22-induced keratinocyte proliferation (**Fig. 6*A***). Consistently, SIRT1-silenced keratinocytes proliferated more actively in response to IL-22 compared to cells transfected with irrelevant siRNA (Fig. 6*A*, bottom graph). Our previous findings showed that IL-22 promoted wound healing in an *in vitro* injury model (5). Therefore, we tested SIRT1 ability to counteract IL-22 also in this system, and found that SIRT1 silencing in keratinocytes greatly enhanced IL-22-induced wound healing 2 d after scratching (Fig. 6*B*). Since the IL-22-induced closure of keratinocyte layer was strictly STAT3-dependent, as assessed by mRNA interference of STAT3 (Fig. 6*B*), it is likely that the negative effects of SIRT1 on IL-22-induced wound healing occurred through STAT3 inhibition.

SIRT1 expression in keratinocytes is down-regulated in psoriatic skin and, *in vitro*, on IFN-γ exposure

STAT3 activity is enhanced in skin affected by psoriasis, especially in response to IL-22, and is responsible for the hyperproliferation and altered differentiation of the psoriatic epidermis (12). Since SIRT1 efficiently controls IL-22-induced activity of STAT3 in keratinocytes, we asked whether SIRT1-STAT3 interplay in psoriasis could be deregulated. Therefore, we first analyzed SIRT1 expression levels in keratinocyte strains obtained from patients with psoriasis, and compared them with keratinocytes isolated from skin of healthy individuals. Keratinocyte cultures were prepared from LS and NLS skin biopsies obtained from the same donor. As shown in **Fig. 7*A***, patients with psoriasis and healthy donors showed similar SIRT1 levels, mainly localized in the cytosol, as assessed by Western blotting. Immunohistochemistry analysis confirmed that SIRT1 was cytosolic and uniformly expressed in keratinocytes of the basal layer of the epidermis (Fig. 7*Bi–iii*). The pattern of SIRT1 expression was identical in healthy and NLS psoriatic skin (Fig. 7*Bi, ii*), which showed also few infiltrating $CD3^+$ T cells (Fig. 7*Biv, v*). Con-

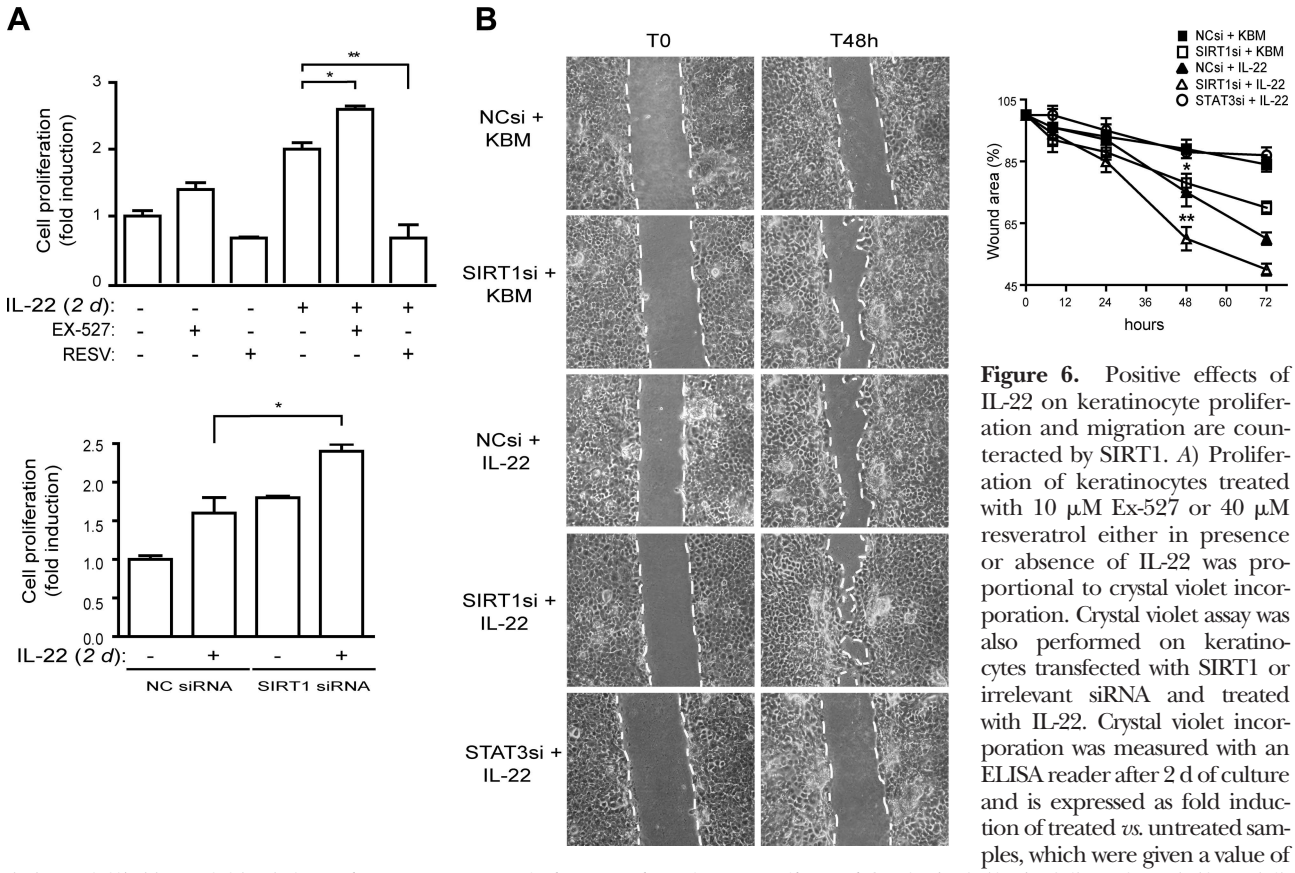

Figure 6. Positive effects of IL-22 on keratinocyte proliferation and migration are counteracted by SIRT1. *A*) Proliferation of keratinocytes treated with 10 μM Ex-527 or 40 μM resveratrol either in presence or absence of IL-22 was proportional to crystal violet incorporation. Crystal violet assay was also performed on keratinocytes transfected with SIRT1 or irrelevant siRNA and treated with IL-22. Crystal violet incorporation was measured with an ELISA reader after 2 d of culture and is expressed as fold induction of treated *vs.* untreated samples, which were given a value of 1. $*P < 0.05$; $**P < 0.01$. *B*) Scratch assays were carried out on keratinocytes silenced for STAT3 (STAT3si) or SIRT1 (SIRT1si) or treated with irrelevant siRNA (NCsi), and incubated or not with 30 ng/ml IL-22 for 48 h. Residual gap between migrating keratinocytes is expressed as percentage of initial scratched area. $*P < 0.01$ *vs.* NCsi samples; $**P < 0.01$ *vs.* IL-22-treated NCsi samples.

versely, basal keratinocytes of LS psoriasis exhibited a reduced and less intense SIRT1 staining if compared to healthy and NLS skin, especially at the tips of the epidermal papillae close to the $CD3^+$ T cell infiltrate (Fig. 7*Biii*, *vi*). Since T cells infiltrating psoriatic lesions produce very high amounts of IFN-γ (*i.e.*, Th1, Th1/Th17 and T cytotoxic cells), we hypothesized that SIRT1 decrement in LS psoriatic keratinocytes could be due to local T-cell-derived IFN-γ. Indeed, treatment of keratinocyte cultures with supernatants obtained from Th1 clones releasing high amounts of IFN-γ (25–35 ng/ml/10^6 cells) reduced SIRT1 protein expression (Fig. 7*C*). Keratinocyte treatments with recombinant cytokines typically present in psoriatic skin confirmed that only recombinant IFN-γ, and not TNF-α, IL-17, or IL-22, could reduce SIRT1 mRNA and protein expression in human keratinocytes (Fig. 7*C*). As a whole, these data demonstrate that although SIRT1 is expressed at comparable levels in keratinocytes from patients with psoriasis and healthy individuals, it is down-regulated in psoriatic epidermis, likely as a result of the local overexpression of IFN-γ.

IFN-γ up-regulates basal STAT3 acetylation, thus reinforcing the IL-22-induced STAT3 phosphorylation and downstream effects in keratinocytes

To see whether the reduced SIRT1 expression by IFN-γ could result in an increase of STAT3 acetylation and, thus, in enhanced STAT3 phosphorylation in response to IL-22, we performed Western blotting experiments on keratinocyte cultures prestimulated with IFN-γ or TNF-α, and then activated with IL-22. As shown in **Fig. 8*A***, IFN-γ significantly enhanced basal STAT3 acetylation in Lys685 (lane 2 *vs.* 1) as well as the IL-22-induced phosphorylation of STAT3 in Tyr705, as compared to treatment with IL-22 alone (lane 5 *vs.* 4). The effect was specific for IFN-γ since TNF-α could not regulate neither basal STAT3 acetylation (Fig. 8*A*, lane 3 *vs.* 1) nor IL-22-induced STAT3 phosphorylation (Fig. 8*A*, lane 6 *vs.* 4). Accordingly, SIRT1 silencing in keratinocytes further enhanced the IFN-γ-induced acetylation in Lys685 of STAT3 (Fig. 8*A*). Concomitantly to the up-regulation of IL-22-induced STAT3 phosphorylation, IFN-γ strengthened IL-22-promoted downstream effects. In particular, IFN-γ significantly enhanced IL-22-induced expression of CXCL8, CXCL1, and SOCS3 mRNA (Fig. 8*B*), as well as IL-22-inhibited accumulation of KRT1 (Fig. 8*D*). Interestingly, nuclear PCNA was strongly reduced by IFN-γ (Fig. 8*C*), which notoriously exerts an antiproliferative effect on keratinocytes (26).

DISCUSSION

Epidermal keratinocytes of psoriatic skin are characterized by activated STAT3, and increased levels of cyto-

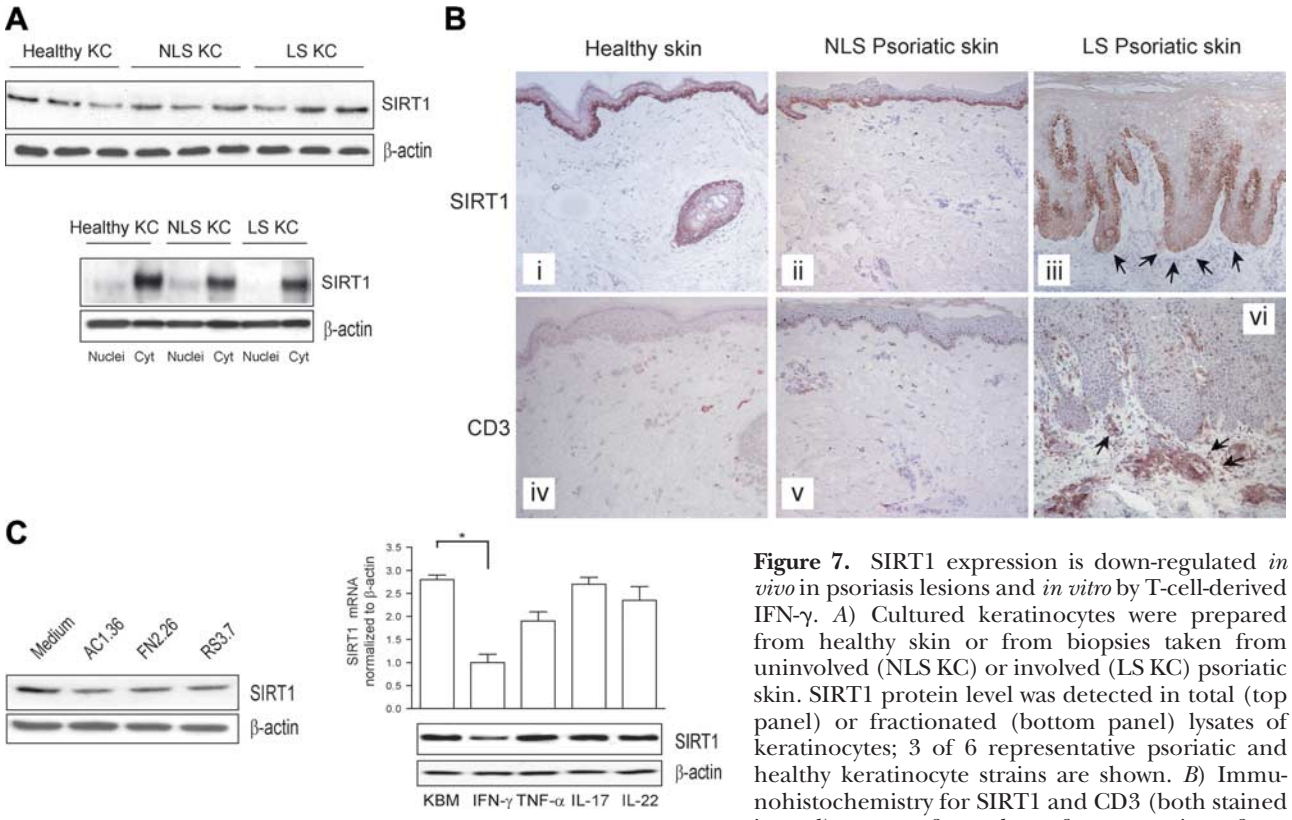

Figure 7. SIRT1 expression is down-regulated *in vivo* in psoriasis lesions and *in vitro* by T-cell-derived IFN-γ. *A*) Cultured keratinocytes were prepared from healthy skin or from biopsies taken from uninvolved (NLS KC) or involved (LS KC) psoriatic skin. SIRT1 protein level was detected in total (top panel) or fractionated (bottom panel) lysates of keratinocytes; 3 of 6 representative psoriatic and healthy keratinocyte strains are shown. *B*) Immunohistochemistry for SIRT1 and CD3 (both stained in red) was performed on frozen sections from biopsies of psoriatic skin including LS (*ii, v*), and NLS (*iii, vi*) areas of plaques. Healthy skin was also analyzed (*i, iv*). Immunoreactivity was revealed by using 3-amino-9-ethylcarbazole as substrate. Arrows (*iii*) indicate area of epidermal papillae where SIRT1 staining was reduced. Representative stainings of 6 psoriatic sections analyzed are shown. *C*) Western blotting of SIRT1 performed on keratinocyte cultures after 24 h activation with diluted (1:3) supernatants derived from 3 Th1 clones (AC1.36, FN2.26, and RS3.7) that produce high amounts of IFN-γ (25–35 ng/ml/10^6 cells). Th1 clones were prepared from peripheral blood of patients with psoriasis; and supernatants were collected after 48 h activation with anti-CD3 and anti-CD28. Alternatively, SIRT1 expression was analyzed by real-time PCR or Western blotting in samples activated with recombinant IFN-γ, TNF-α, IL-17 or IL-22 (right panel). Western blots are representative of 2 independent experiments performed on 2 different keratinocyte strains. $^*P < 0.01$.

kines and growth factors that promote STAT3 activation have been found within psoriatic lesions (12, 27). Among them, IL-22 potently triggers STAT3, which has a pathogenetic role by altering the proliferative and differentiation processes in keratinocytes, and inducing inflammatory molecules (9). STAT3 proper activation requires phosphorylation in Tyr705 and in Ser727 residues but also Lys685 acetylation (13, 28). Recently, Nie *et al.* (16) have described the involvement of SIRT1 in promoting STAT3 Lys685 deacetylation, and, thus, in down-regulating STAT3 phosphorylation and activity in epatocytes during gluconeogenesis. STAT3 acetylation is rarely found in resting cells but it can be efficiently induced by cytokines, such as oncostatin M, IL-6, and by type I IFNs (15). In this study, we showed that STAT3 acetylation in Lys685 is constitutive in human keratinocytes, and negatively controlled by SIRT1. STAT3 acetylation in Lys685 was also found to be modulated by IFN-γ, which strongly induced acetylated STAT3 by repressing SIRT1 expression in keratinocytes. SIRT1 inhibition by IFN-γ was an event occurring also *in vivo*, especially in skin diseases characterized by an infiltrate of IFN-γ-releasing T cells. For instance, psoriasis showed a decrease of SIRT1 expression in basal layer keratinocytes, especially at the tips of the epidermal papillae close to the $CD3^+$ T-cell infiltrate. However, the constitutive expression of SIRT1 was similar in psoriatic and healthy cultured keratinocytes as well as in healthy and NLS psoriatic skin, indicating that the impaired SIRT1 expression was the consequence of the microenviromental cytokine milieu and not an intrinsic defect of the psoriatic epidermis. SIRT1 inhibition by IFN-γ leading to the enhancement of Lys685 acetylation of STAT3 could represent a central molecular event reinforcing keratinocyte responses to IL-22. In fact, IFN-γ pretreatment of keratinocyte cultures strongly enhanced STAT3 phosphorylation in Tyr705 and STAT3-dependent gene expression induced by IL-22. The amplificatory effect of IFN-γ on IL-22 signaling in keratinocytes is analogous to that promoted by TNF-α, even if the molecular mechanisms and genes regulated by the combination of IL-22 and TNF-α are completely different (5). The specificity of IFN-γ and TNF-α effects on IL-22 signaling could depend on their ability to regulate and activate different molecular pathways. Concerning STAT3 signaling, TNF-α, differently from IFN-γ, did not affect the phosphorylation of STAT3 nor its acetylation. Other than potentiating

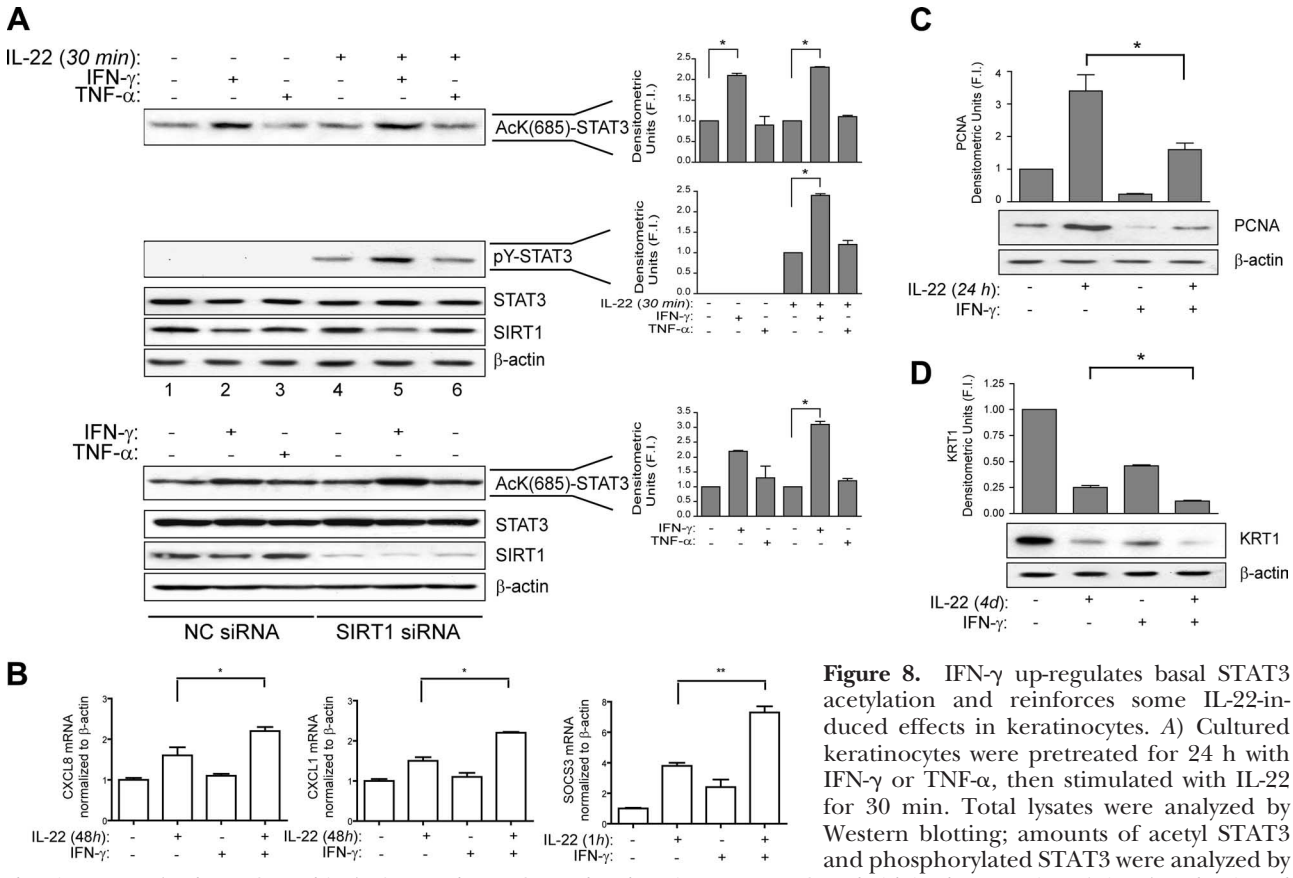

Figure 8. IFN-γ up-regulates basal STAT3 acetylation and reinforces some IL-22-induced effects in keratinocytes. *A*) Cultured keratinocytes were pretreated for 24 h with IFN-γ or TNF-α, then stimulated with IL-22 for 30 min. Total lysates were analyzed by Western blotting; amounts of acetyl STAT3 and phosphorylated STAT3 were analyzed by densitometry. Analyses of acetyl STAT3 were also performed on keratinocytes transfected with irrelevant or SIRT1 siRNA and activated for 24 h with IFN-γ or TNF-α. *B*) CXCL8, CXCL1, and SOCS3 mRNA expression was evaluated by real-time PCR in cultured keratinocytes treated with IL-22 and/or IFN-γ. *C*) Nuclear PCNA was analyzed in keratinocytes treated with IL-22 and/or IFN-γ for 24 h. *D*) KRT1 expression analysis was performed on keratinocyte cultures undergoing terminal differentiation or stimulated with IL-22 and/or IFN-γ for 4 d. Variations of PCNA and KRT1 levels were also determined by densitometry. Western blots are representative of 2 independent experiments performed on 2 different keratinocyte strains. $*P < 0.05$; $**P < 0.01$.

IL-22 signaling in keratinocytes, IFN-γ was also able to counteract IL-22-induced downstream effects in keratinocytes. In fact, it could reduce both basal and IL-22-promoted PCNA nuclear accumulation, accordingly to its potent antiproliferative effect on this cell type. Therefore, IFN-γ can either potentiate or inhibit IL-22 signaling, with this dual effect depending on the IFN-γ ability to induce, in addition to STAT3 acetylation, other molecular cascades. Among these, STAT1 activation, usually associated with a decrease in proliferation rate in IFN-γ-sensitive cells, could be responsible for the dominant inhibitory effect of IFN-γ on IL-22-induced PCNA expression. It would be important to evaluate whether IFN-γ has a prevailing role also on IL-22-induced proliferation in keratinocytes, and whether psoriatic keratinocytes are less or more sensitive than healthy cells to the antiproliferative or mitogenic effect of IFN-γ and IL-22, respectively. Indeed, previous studies have already shown that psoriatic keratinocytes aberrantly respond to IFN-γ, whose injection into uninvolved psoriatic skin causes epidermal hyperplasia and plaque development (29). The enhanced proliferative response of psoriatic keratinocytes to IFN-γ has been supposed to rely on a reduced activation of STAT1 signaling and on an altered expression level of the IFN-γ receptor complex in the epidermis of psoriatic lesions compared to healthy skin (30).

The SIRT1 inhibitory effect on STAT3 acetylation in Lys685 influenced only the IL-22-induced phosphorylation in Tyr705, and not in Ser727 of STAT3, as assessed by silencing SIRT1 expression. In addition, SIRT1 could not modulate IL-22-dependent ERK1/2 phosphorylation, indicating the selectivity of SIRT1 action on STAT3. Indeed, it is well known that SIRT1 can regulate the acetylation status and, thus, the phosphorylation and function of several transcription factors, including p53, E2F1, NF-κB, c-Jun, and members of the FoxO family (31). Modification of STAT3 acetylation in keratinocytes by manipulating SIRT1 expression confirmed the fundamental role of SIRT1 in regulating the IL-22-induced STAT3 activity. In fact, while SIRT1 depletion enhanced STAT3 transacting capability, its overexpression inhibited STAT3 function in IL-22-treated keratinocytes. As a direct consequence of enhanced STAT3 activity, SIRT1-depleted keratinocytes were more sensitive than control cells to the effects of IL-22, which could better induce CXCL8, CXCL1, and SOCS3 mRNA expression as well as nuclear translocation of PCNA, cyclin D1, and pRB. SIRT1-silencing and, thus, STAT3 activity enhancement in keratinocytes also resulted in an increased reduction of KRT1 and loricrin

expression. In addition, SIRT1 influenced the IL-22-induced biological effects on keratinocytes, since it could significantly inhibit the mitogenic and migratory activities of IL-22 in these cells. In particular, enzymatic activation of SIRT1 by resveratrol and inhibition by Ex-527 could reduce and enhance, respectively, the IL-22-induced keratinocyte proliferation. Also, SIRT1-silenced keratinocytes proliferated more actively in response to IL-22 compared to control cells, and showed enhanced IL-22-induced wound healing. Since the IL-22-induced closure of keratinocyte layer was strictly dependent on STAT3, it is possible that the negative effects of SIRT1 on IL-22-induced wound healing were explicated through the inhibition of STAT3 acetylation. It would be interesting to analyze whether, similarly to psoriasis, SIRT1 keratinocyte expression is impaired during skin wound healing processes. This hypothesis is in line with the observations that keratinocytes of both psoriasis and cutaneous wound healing sites are both oriented toward a robust regenerative program in response to IL-22 and display a strong STAT3 activation driving tissue regeneration and protection (5, 9). SIRT1 inhibitory function on proliferative processes of keratinocytes is consistent with previous data demonstrating that SIRT1 is an inhibitor of keratinocyte proliferation and an inducer of keratinocyte differentiation. SIRT1 inhibition of keratinocyte proliferation could occur through a mechanism involving cell cycle regulators, such as E2F1 (17). Therefore, the decrement of the prodifferentiation activity by SIRT1 in psoriatic keratinocytes could be responsible for the altered phenotype of psoriatic epidermis, which is characterized by a reduction of the differentiated cell compartment with respect to the germinative layer (32).

In this study, we showed that SIRT1 was constitutively expressed by keratinocytes and efficiently contributed to STAT3 deacetylation in these cells (**Fig. 9**). During inflammatory skin processes characterized by a concomitant presence of IFN-γ and IL-22, IFN-γ strongly down-regulated the keratinocyte expression of SIRT1 and IL-22 activated STAT3. SIRT1 decrement determined an accumulation of STAT3 acetylated in Lys685, thus favoring STAT3 phosphorylation in Tyr705 and keratinocyte responses to IL-22. Among molecules regulated by IL-22-induced STAT3 and influenced by SIRT1, PCNA, cyclin D1, and pRB play a fundamental role in proliferation, a biological process robustly induced by IL-22 in keratinocytes during proliferative skin disorders, such as psoriasis. In psoriatic lesions, SIRT1 inhibition could be responsible for the strong activation of STAT3 and, thus, for the exaggerated responses of epidermal keratinocytes to IL-22 and other proinflammatory cytokines signaling through STAT3 (*i.e.*, IL-6 and oncostatin M) (15, 33). Therefore, the use of activators of SIRT1 decreasing STAT3 acetylation in epidermal keratinocytes may be therapeutically relevant for the treatment of psoriasis but also for other skin diseases characterized by aberrant STAT3 activation. The latter include cutaneous tumors, such as papilloma and squamous cell carcinoma, where STAT3 has a role in the tumor promotion stage of epithelial carcinogenesis (34). Indeed, STAT3 inhibition by STAT3 decoy oligonucleotides in mice harboring activated STAT3 abrogated the *de novo* generation of tape stripping-induced psoriatic lesions in mice overexpressing STAT3 in the epidermis as well as the development of TPA-induced skin carcinoma (12, 27, 34). In addition, the STAT3 inhibitor STA-21 molecule, an antibiotic which blocks STAT3 dimerization and DNA binding, has been successfully employed in a pilot study for the treatment of psoriasis (35). Further investigation will be required to unveil the effects of SIRT1 and STAT3 interplay in keratinocytes, and mainly the outcome of an efficient manipulation of SIRT1 activity in the epidermis that will ultimately allow development of novel therapies for STAT3-dependent skin disorders. FJ

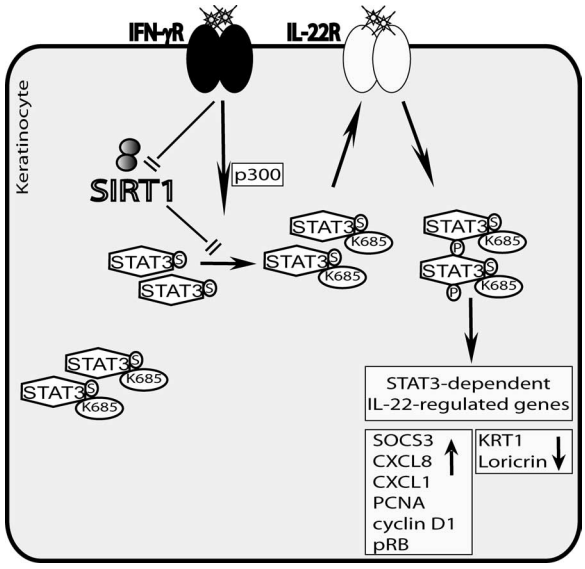

Figure 9. Schematic model of regulation of IL-22-induced signaling of STAT3 in human keratinocytes by IFN-γ *via* SIRT1 inhibition. In human keratinocytes, STAT3 is constitutively acetylated in Lys685, as a result of a tight balance between acetylation and deacetylation processes executed respectively by histone acetyl transferase (HAT) and deacetylase enzymes, including SIRT1. STAT3 is also constitutively phosphorylated in Ser727 residue in keratinocytes, although it cannot efficiently transactivate STAT3-responsive genes. During inflammatory skin responses, T-cell-derived IFN-γ strongly down-regulates the keratinocyte expression of SIRT1, which can no longer deacetylate STAT3. The consequent accumulation of STAT3 acetylated in Lys685 favors keratinocyte responses to IL-22, and, in particular, STAT3 phosphorylation in Tyr705, a post-translational modification fundamental for STAT3 activity on target genes. Among molecules regulated by IL-22-induced STAT3 and influenced by SIRT1, PCNA, cyclin D1, and pRB play a fundamental role in proliferation, a biological process robustly induced by IL-22 in keratinocytes in proliferative skin disorders, such as psoriasis.

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Received for publication September 20, 2010.
Accepted for publication November 11, 2010.