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Mammalian target of rapamycin in inflammatory skin conditions

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Introduction

The conserved serine/threonine kinase mammalian target of rapamycin (mTOR) is a major regulator of survival growth, proliferation and motility in response to mitogens, energy as well as nutrient levels, exerting its effects through two distinct signaling complexes, known as mTOR complex (mTORC) 1 and mTORC2 (1, 2). In particular, the rapamycin-sensitive mTORC1 promotes cell growth and proliferation whereas mTORC2, rapamycin-non sensitive, is involved in the regulation of cell polarity and in the functional phosphorylation of cytoskeleton actin fibres (3-5). Therefore, mTOR functions as a central node in a complex network of signaling pathways that are involved both in normal physiological, as well as pathogenic events (6). mTOR signaling occurs in concert with upstream phosphoinositide-3-OH kinase (PI3K)/Akt and tuberous sclerosis complex (TSC) and several downstream effectors (7). Because of its central role in many different cellular activities, mTOR dysregulation can be involved in a great number of either inflammatory or neoplastic conditions through the coordinated phosphorylation of proteins that directly regulate cell-cycle progression and metabolism, as well as transcription factors that regulate the expression of genes involved in the oncogenic processes (6). The importance of mTOR signaling in oncology is now widely accepted, and agents that selectively target mTOR have been developed as anticancer drugs (8-11). Moreover, mTOR functions are also reported to be altered in metabolic disorders, such as obesity (5) and type 2 diabetes (12) as well as in autoimmune disorders, such as rheumatoid arthritis (13), inflammatory bowel disease (14) and lupus erythematosus (15). Recently, mTOR pathway was shown to play a role in regulating the immune response, not only in myeloid cells, but also in keratinocytes (16), potentially contributing to cytokines production in skin inflammation (17). Indeed, mTOR involvement in some of the most common inflammatory dermatoses has now been demonstrated (18).

Chapter I

mTOR

mTOR structure

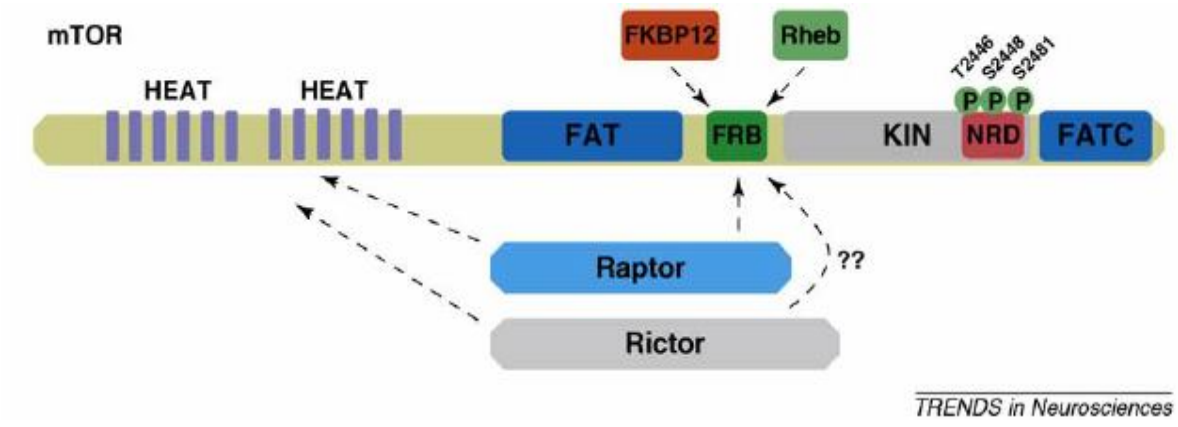
mTOR was identified and cloned almost 20 years ago (1, 2, 19) after the discovery of the two yeast genes, TOR1 (target of rapamycin) and TOR2, in the budding yeast *Saccharomyces cerevisiae* during a screen for resistance to the immunosuppressant drug rapamycin (20, 21). Rapamycin, a macrocyclic lactone produced by *Streptomyces hygroscopicus*, was first isolated from a soil sample of Easter Island (Rapa Nui) during a program for anti-microbial agents in the early 1970s, and subsequently discovered to have equally potent immunosuppressive and anti-tumor properties (22-26). mTOR, also known as FRAP (FKBP12-rapamcyin-associated protein), RAFT1 (rapamycin and FKBP12 target), RAPT 1 (rapamycin target 1), or SEP (sirolimus effector protein), is an ubiquitous and constitutively expressed serine/threonine kinase of 289 kDa (19, 27), member of the PI3K-kinase-related kinase (PIKK) superfamily since its C terminus shares strong homology to the catalytic domain of the lipidic kinase PI3K (19). However, there is no experimental evidence that it displays lipid kinase activity, and in this respect, it is similar to other protein kinases such as ATM and ATR (28).

C-terminal end of mTOR includes the kinase catalytic domain (KIN). The KIN domain also presents a small region, called negative regulatory domain (NRD), that is probably a site of phosphoregulation (7, 29). Within this region, phosphorylation at thr2446, ser2448 and ser2481 are correlated with overall higher levels of mTOR activity. Thr2246 is targeted by AMPK and S6K, ser2448 is a target of Akt and S6K, whereas ser2481 is an autocatalytic target of mTOR (30-33). Some of these residues are autophosphorylated even in the presence of rapamycin, whereas others are substrates of the downstream

effectors of mTOR itself, thereby providing multiple mechanisms for feedback regulation (7, 29).

Immediately upstream of the catalytic domain is the FRB (FKBP12–rapamycin binding) domain, the site of inhibitory interaction between rapamycin and mTOR. Rapamycin bound to FK506 binding protein 12 (FKBP12) disrupts protein–protein interactions that are key to mTOR function (34). The FRB is also involved in the interaction between mTOR and other mTOR complex members including Raptor and Ras homolog enriched in the brain (Rheb). In addition, mTOR contains a relatively large FAT domain, which is also present in other PIKK proteins (35). A second FAT domain (FATC) is located at the distal C-terminal end of mTOR. Both FAT domains are absolutely necessary for mTOR catalytic function and the deletion of even a single amino acid from this domain abrogates the activity (36, 37).

The N-terminal portion of mTOR contains 20 tandem HEAT (Huntignton, EF3, A subunit of PP2A, TOR1) repeats. Each HEAT repeat consists of two α-helices of 40 amino acids, each with a specific pattern of hydrophobic and hydrophilic residues. Tandem HEAT repeats are present in many proteins and are implicated in protein–protein interactions (38).



mTOR complexes

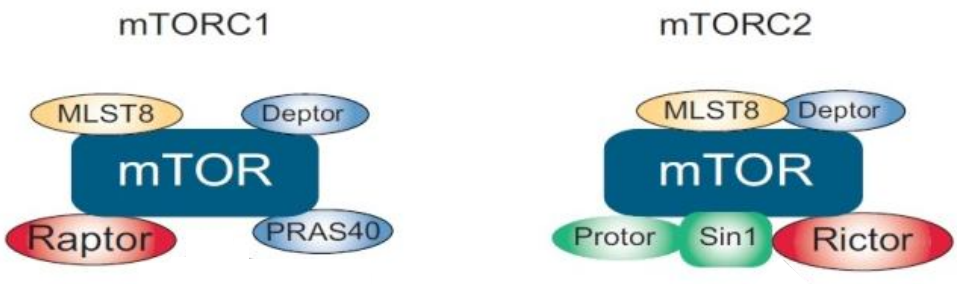
Cumulative evidence indicates that mTOR acts as a “master switch” of cellular anabolic and catabolic processes, regulating the rate of cell growth and proliferation by its ability to sense mitogen, energy and nutrient levels (39, 40). mTOR functions as two distinct signalling complexes known as *mTOR complex* (mTORC) *1* and *mTORC2* (30). These complexes share some common constituents such as the G-like protein family member (**mLST8**) and DEP-domain-containing mTOR-interacting protein (**Deptor**) but can be largely distinguished by their unique components (3, 4, 29).

The rapamycin and nutrient-sensitive complex, mTORC1, consisting of mTOR, mLST8 and **Raptor** (regulatory associated protein of mTOR) was first reported in 2002 (3, 41). The main function of mTORC1 is to regulate cell growth, proliferation and survival by sensing mitogen, energy and nutrient signals (42). Raptor acts as a scaffolding companion to mTOR, binding TOR signaling (TOS) motif-containing proteins and shuttling them to the mTOR catalytic domain (3, 7). mTORC1 is sensitive to rapamycin via competition between Raptor and FKBP12–rapamycin for binding to the FRB domain (3). In addition to Raptor, mTORC1 contains other proteins such as proline-rich Akt/PKB substrate 40 kD (**PRAS40**). PRAS40 regulates mTOR–Raptor interactions and negatively regulates mTOR signaling by blocking mTORC1 access to its substrates (43). However, in another study, PRAS40 was required for substrate activation, suggesting that in some contexts facilitates mTORC1 function. The interaction between mTOR and PRAS40 is disrupted by rapamycin, indicating that FKBP12 can mediate its nascent inhibitory activity (43-45).

mTORC2, the second distinct mTOR complex, was identified in 2004 (4, 29). It was originally thought to be rapamycin-insensitive (4). However, further studies demonstrated that prolonged rapamycin treatment inhibits the assembly and function of mTORC2 in some cell lines as well (46). mTORC2 regulates the actin cytoskeleton by mediating

phosphorylation state of protein kinase C α (PKCα) (4), and modulates cell survival in response to growth factors by phosphorylating its downstream effector Akt (protein kinase B, PKB) at the hydrophobic motif site, S473 (29, 47, 48). Like mTORC1, mTORC2 also includes mTOR and mLST8, but instead of Raptor, mTORC2 contains two special subunits, **Rictor** and mammalian stress-activated protein kinase (SAPK)-interacting protein 1 (**mSin1**) (4, 29, 49). Sin1 encodes an essential function because the deletion of Sin1 is embryonically lethal (50). Another recently identified mTORC2 component is protein observed with Rictor (**Protor**). Protor binds Rictor independently of mTOR and does not appear to be required for mTORC2-mediated Akt activation (50-53). In addition, PRR5 (proline-rich protein 5) and Hsp70 are other novel components of mTORC2 (54-57).

Both mTOR complexes function predominantly in the cytoplasm. However, experiments using a nuclear export receptor inhibitor indicate that mTOR may actually be a cytoplasmic-nuclear shuttling protein. This nuclear shuttling appears to play a role in the phosphorylation of mTORC1 substrates induced by mitogenic stimulation and in the consequent upregulation of translation (58). This dual subcellular localization was also demonstrated by immunohistochemical analysis (IHC) in a study on human carcinomas.



Upstream regulators of mTOR

As described above, mTOR integrates various signals to regulate cell growth and proliferation. A number of biologically important stimuli have been shown to induce mTOR signaling: growth factors, nutrients (amino-acid, glucose and oxygen etc.), energy and stress (59).

For example, binding of insulin or insulin-like growth factor (IGF) to its receptor (IGFR) leads to the recruitment and phosphorylation of insulin receptor substrate (IRS). IGFR and IRS then interact with PI3K through specific phosphorylated tyrosine residues which lead to the activation of mTOR (7). PI3K phosphorylates phosphatidylinositol-4-5-bis-phosphate (PIP2) to form phosphatidy-linositol-3-4-5-trisphosphate (PIP3), which binds to Akt as well as PDK1 and facilitates their relocalization to the membrane. Colocalization of Akt with PDK1 results in the partial activation of Akt through phosphorylation at thr308 (60). Full activation of Akt requires additional phosphorylation at ser473 by the putative kinase PDK2, which includes mTORC2 complex, mitogen-activated protein kinase (MAPK)-activated protein kinase and other kinases (7, 36, 61, 62). Thus, mTORC2 performs a positive feedback role in the activation of Akt, and could thereby indirectly activate mTORC1. Akt suppresses the activity of the downstream TSC1/2 complex which otherwise inhibits the activity of Rheb (7). This TSC1/2 complex functions as a key player in the regulation of the mTOR pathway by mediating inputs from the PI3K/PTEN/Akt and Ras/Erk1/2 signaling pathways, and by regulating translation initiation in response (63). Activated Erk1/2 directly phosphorylates TSC2 at ser664, (and possibly ser1798 as well) (49, 64). This site differs from those phosphorylated by Akt (ser939, the1462 and possibly additional sites) (65), but either causes functional inactivation of TSC1/2. Of note, TSC2 is also a substrate of S6K (59). Conversely, PIP3 accumulation is antagonized by the lipid phosphatase PTEN

(phosphatase and tensin homolog deleted on chromosome 10), which converts PIP3 to PIP2 (63, 66). Therefore, one critical outcome of PTEN inactivation is an increase in mTOR activity (63). Rheb, in turn, binds directly to the kinase domain in mTOR and drives the formation of that mTOR-Raptor complex in a GTP-dependent manner (67, 68). Other mTOR activators are amino acids, in particular, leucine, which enhances mTORC1 activation via inhibition of TSC1/2 or via stimulation of Ras homologue enriched in brain (Rheb), which is a small GTPase required for mTOR activation (7, 69).

Moreover, mTORC1 indirectly senses the energy status of the cell through the liver kinase (LK) B1-mediated pathway, which functions in parallel to the PI3K pathway. LKB1, a tumor suppressor inactivated in Peutz-Jeghers syndrome, activates AMP-activated kinase (AMPK) in response to energy deprivation (66). This activation of AMPK in response to low cellular energy (high AMP/ATP ratio) downregulates energetically demanding processes, such as protein synthesis, and stimulates ATP-generating processes. Activated AMPK also phosphorylates and activates TSC2 by enhancing its GAP activity, resulting in the inhibition of mTORC1 (66).

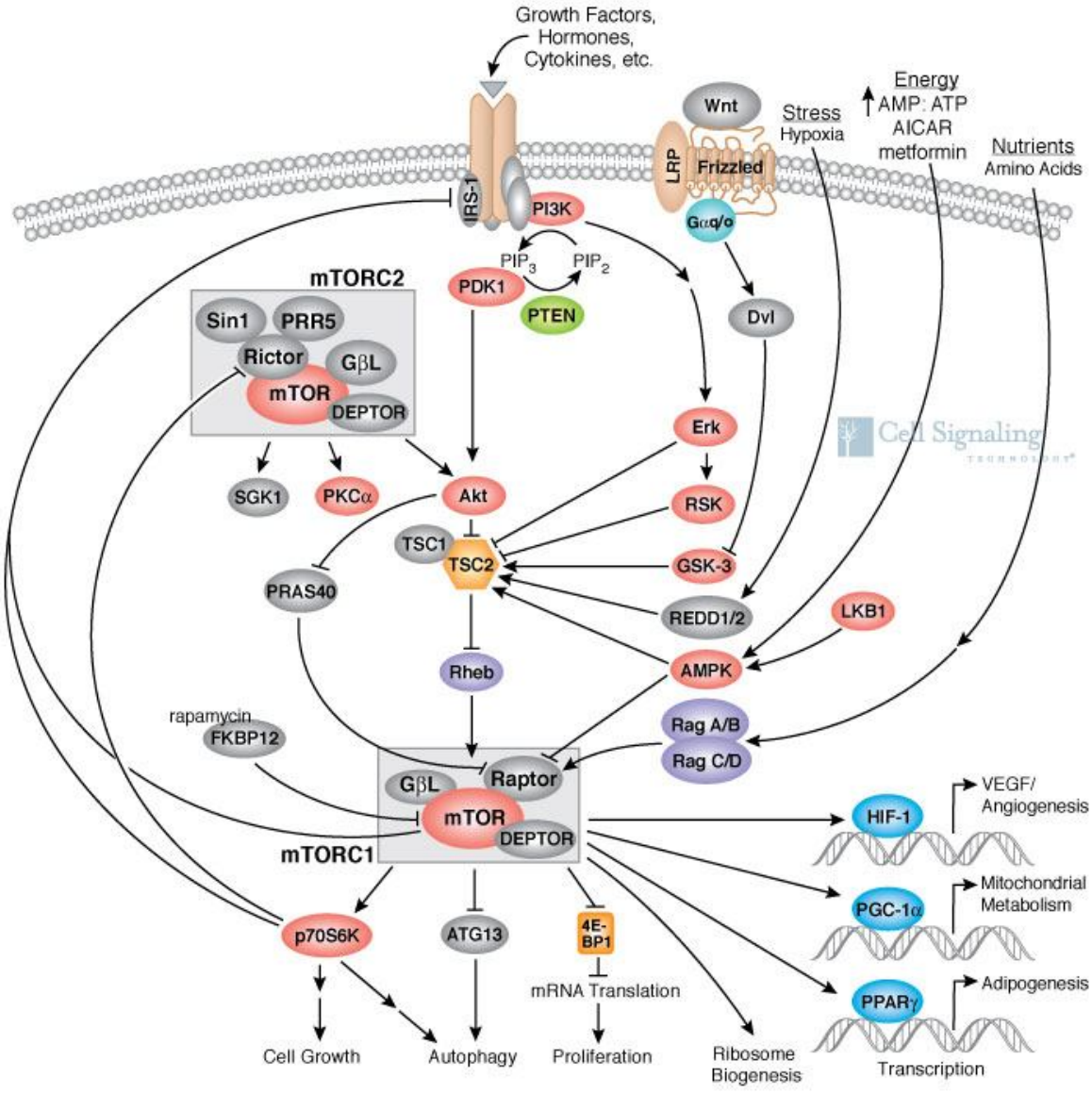
Lastly, mTOR activity is repressed not only under conditions of energy deprivation, but also conditions of stress, such as hypoxia, heat shock, and low cellular energy state (70). The hypoxic signal is transduced to mTORC1 via two homologous proteins REDD1 and REDD2, which are upregulated by HIF-1α. REDD acts downstream of PI3K and functions to inhibit mTORC1 signaling (70).

Downstream targets of mTOR

The major targets of mTOR are components of the translation machinery, and in particular, those responsible for ribosome recruitment to mRNA (71). Indeed, translation is regulated in most instances at the step during which a ribosome is recruited to the 5'end of an mRNA, positioned at a start codon (72). The 5'end of all nuclear-transcribed mRNAs possess a cap structure (m7GpppN, in which "m" represents a methyl group and "N", any nucleotide) that is specifically recognized by eukaryotic translation initiation factor 4E (eIF4E). eIF4E is part of the pre-initiation complex eIF4F containing the large scaffolding proteins eIF4GI and eIF4GII and the RNA helicase eIF4A (72-74). Following its binding to the 5'cap, eIF4F unwinds the mRNA 5'-proximal secondary structure and, in association with several other initiation factors, allows the binding of the 40S ribosomal subunit (72). Strikingly, several components of the ribosome recruitment machinery as well as ribosomal components are either direct or indirect targets of mTOR (71). In mammals, **S6Ks** and **4E-BPs** are the best characterized downstream targets of mTOR. 4E-BPs, a family of translational repressor proteins, consists of three low molecular weight proteins, 4EBP1, 4E-BP2, and 4E-BP3 (75-77). 4E-BP1 is directly phosphorylated by mTORC1. Hypophosphorylated 4E-BP1 binds tightly to eIF4E, the mRNA cap-binding protein, and represses cap-dependent translation by blocking the binding of eIF4E to eIF4G. In response to sufficient growth factors and nutrients stimulation, seven sites (Thr 37, Thr 46, Ser 65, Thr 70, Ser 83, Ser 101 and Ser 112) of 4E-BP1 can be phosphorylated (78). The phosphorylation of 4E-BP1 at multiple site results in its dissociation from eIF4E, allowing eIF4E to engage eIF4G (76, 79). eIF4G serves as a scaffold protein for the assembly of other initiation factors including eIF4A, which acts as an ATP-dependent RNA helicase, and further interacts with eIF3, which recruits the 40S ribosome to the 5'end of the mRNA (80).

Mammalian cells contain two similar S6 kinase proteins, S6K1 and S6K2 (81). S6K2, which has 70% overall amino acid identity with S6K1, was discovered much later than S6K1 (82). Both the activation of S6K1 and S6K2 are regulated by mTOR (83, 84). S6K1 is known as the major ribosomal protein S6 (rpS6) kinase in mammalian cells and is pointed as a key player in the control of cell growth (cell size) and proliferation (85-87). Early studies suggested that activated S6K1 regulates protein synthesis through phosphorylation of the 40S ribosomal protein S6, and this was thought to increase the translational efficiency of a class of mRNA transcripts with a 5'-terminal oligopolypirymidine (5'-TOP) (88, 89). These mRNAs encode exclusively for components of the translation machinery, including all ribosomal proteins, elongation factors, and poly(A)-binding protein (PABP). Critical data supporting the idea that S6K1 is required for 5'-TOP mRNA translation include the demonstration that a rapamycin-resistant S6K1 mutant confers rapamycin resistance to the translation of 5'-TOP mRNAs (89). As an alternative to S6, eIF4B is a physiologically relevant target of S6K1 that could explain its effect on translation and cell growth. As stated above, eIF4B is required for efficient recruitment of ribosomes to mRNA (73). eIF4B is an RNA-binding protein that specifically stimulates the ATPase and RNA helicase activities of eIF4A (90). eIF4B is phosphorylated in response to a variety of extracellular stimuli, such as serum, insulin, and phorbol esters that promote cell growth and proliferation (91).

In addition, mTOR is also involved in the regulation of some proteins including CLIP-170 (cytoplasmic linker protein-170) (92), eukaryotic elongation factor 2 (eEF2) kinase (93), ornithine decarboxylase (ODC) (94), glycogen synthase (95), hypoxia-inducible factor 1α (HIF-1α) (96, 97), lipin (98) PKCδ and PKCε (99) protein phosphatase 2A (PP2A) (100), p21Cip1 and p27Kip1 cyclin-dependent kinase inhibitors (101, 102), retinoblastoma protein (Rb) (103), and signal transducer and activator of transcription 3 (STAT3) (104).


Growth Factors,
Hormones,
Cytokines, etc.
Wnt
Stress
Hypoxia
Energy
AMP: ATP
AICAR
metformin
Nutrients
Amino Acids
LRP
Frizzled
IRS-1
PI3K
Gαq/o
PIP3
PIP2
PDK1
PTEN
Dvl
mTORC2
Sin1
PRR5
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HIF-1
VEGF/
Angiogenesis
PGC-1α
Mitochondrial
Metabolism
PPARγ
Adipogenesis
p70S6K
ATG13
4E-
BP1
mRNA Translation
Cell Growth
Autophagy
Proliferation
Ribosome
Biogenesis
Transcription

Chapter II

mTOR and diseases

mTOR and disease

Because of its central role in cellular functions, mTOR dysregulation is involved in a number of either inflammatory or neoplastic conditions. In support of this, the importance of mTOR in the immune system has long been recognized through the finding of the immunosuppressive role of rapamycin. Indeed, rapamycin is used to prevent post-transplantation allograft rejection in kidney transplant recipients, because, inhibiting mTOR, it strongly suppresses interleukin-2 (IL-2) stimulated T cell proliferation (105). Moreover, regulatory T cells (Tregs) induction is inhibited by mTOR activation and is enhanced by rapamycin (106), in fact, in mTOR knockout mice Treg phenotype is enhanced whereas T helper cells 1 (Th1), Th2 and Th17 lineages differentiation is abrogated (107). Furthermore, in many recent studies, other than T-cells, also dendritic cells (DCs) functions have been shown influenced by mTOR signalling pathways. *In vivo* or *in vitro* rapamycin administration can impair maturation and differentiation of DCs which remain tolerogenic and fail to upregulate co-stimulatory molecules (108, 109).

mTOR is frequently dysregulated in cancer and its inhibitors, temsirolimus and everolimus, are used in patients with renal cell carcinoma who had poor prognosis or had failed prior therapies (8, 9). Temsirolimus was reported to be efficacious in treatment of refractory mantle cell lymphoma (10), whereas everolimus was recently reported to increase progression-free survival in patients with advanced pancreatic neuroendocrine tumours (11). However, despite isolated successes in subsets of cancer, the performance of mTOR inhibitors has been undistinguished, suggesting that the full therapeutic

potential of targeting mTOR has yet to be exploited. Moreover, mTOR functions are reported to be altered in metabolic disorders, such as obesity (105) and type 2 diabetes (12) as well as in inflammatory and autoimmune disorders such as rheumatoid arthritis (13), inflammatory bowel disease (14) and lupus erythematosus (15). The mTOR pathway has been shown to play a role in regulating the immune response, not only in myeloid cells but also in keratinocytes (16) and potentially contributes to cytokine production in psoriasis (17). As psoriasis is also considered a hyper-proliferative disorder, thus requiring enhanced cell growth, mTOR signalling could be activated in psoriatic lesions suggesting a possible role in the pathogenesis of the disease. Recent studies have also hypothesized a possible role for mTORC1 in acne pathogenesis (110). Acne is an inflammatory process involving the pilosebaceous unit, with a prevalence rates of over 85% in adolescents (111). Western diet (WD), characterized by high glycemic load and high dairy protein consumption, has been suggested to be a fundamental acne promoting factor (112, 113). High milk and dairy intake, as well as hyperglycemia, determine an over-stimulation of insulin and insulin-like growth factor (IGF)-1 mediated signals (114); this results in Akt phosphorilation with inhibition of TSC1 and 2, negative upstream regulators of mTOR, and consequent mTORC1 and mTORC2 activation (115). mTORC1 activates sterol regulatory element-binding protein (SREBP), the master transcription factor of lipogenesis, and increases androgen hormone secretion (116). mTORC1, directly activated by testosterone, amplifies androgen signaling of sebaceous follicles (110). Indeed, testosterone is able to increase the phosphorylation of mTOR and its major downstream effectors eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) as well as ribosomal protein S6 kinase beta-1 (S6K1) (117).

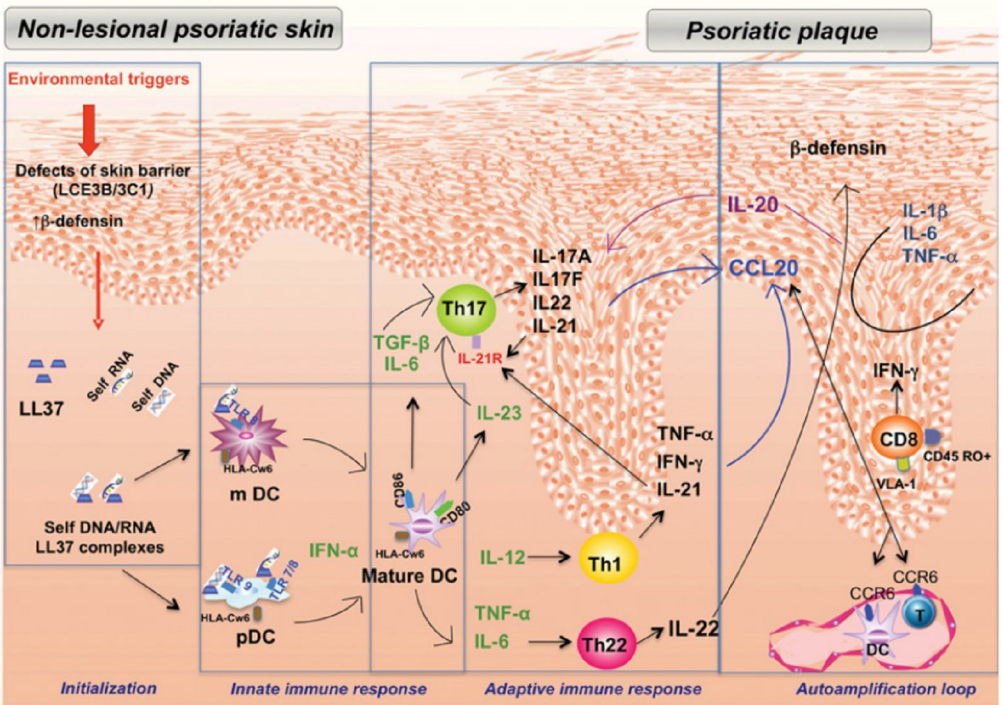
Pathogenesis of psoriasis

Psoriasis is a complex multifactorial disease, where various environmental triggers (e.g. trauma, stress, infections and drugs) promote, in genetically predisposed individuals, the activation of an exaggerated and poorly controlled immuno-inflammatory response in the skin characterized by hyperplasia of epidermal keratinocytes, vascular hyperplasia and ectasia, infiltration of T lymphocytes, neutrophils and other types of leucocytes. Therefore, rather than viewing psoriasis as a disease caused by a single cell type or a single inflammatory cytokine, it is probably best to conceptualize its pathogenesis as linked to many interactive responses between infiltrating leucocytes, resident skin cells, and an array of proinflammatory cytokines, chemokines, and chemical mediators produced in the skin under regulation of the cellular immune system. Dendritic cells (DCs), the most potent APCs (antigen-presenting cells), are sentinels of the immune system. In normal skin, DCs are found in the epidermis [LCs (Langerhans cells)] and dermis (myeloid and plasmacytoid DCs). LCs reside in the suprabasal layers of the epidermis in close contact with keratinocytes. After activation, LCs up-regulate chemokine receptors on their surface and migrate to skin-draining lymph nodes, where they present antigenic peptides they have encountered in the skin to naïve T-cells. The mobilization of LCs into draining lymph nodes in response to stimuli that normally induce migration [e.g. chemical allergen, TNF-α and IL -1β] is largely absent in psoriasis (118) These findings raise the possibility that LCs retained within the epidermis present antigens locally and exacerbate the ongoing inflammatory reaction. In the inflamed dermis of psoriatic patients, there is a marked increase in myeloid CD11c+ DCs. These cells, probably derived from circulating DC precursors, migrate into the skin in response to chemotactic signals and synthesize high levels of pro-inflammatory cytokines (e.g. IL-12 and IL-23) (119). The dermal CD11c+ DCs which make TNF-α and iNOS (inducible

nitric oxide synthase) (TIP-DCs) are thought to be the human equivalent of a similar DC subset which is needed for the clearance of some pathogens in mice. There are also increased numbers of plasmacytoid DCs in psoriatic skin compared with normal skin. These express TLR (Toll-like receptor) 9 and produce large amounts of IFN (inteferon)-α when activated with the microbicidal cathelicidin LL37 bound to self-DNA fragments released by stressed or dying cells in the skin (Figure 1) (120). Plasmacytoid DC also express TLR8 and make IFN-α when stimulated with self-RNA–LL37 complexes. LL37 can also bind self-RNA released by dying cells, and these complexes activate TLR7 in plasmacytoid DCs and, like self-DNA–LL37 complexes, trigger the secretion of IFN-α. In contrast with self-DNA–LL37 complexes, self-RNA–LL37 complexes can interact with TLR8 on classical myeloid DCs and promote their differentiation into mature DCs with secretion of TNF-α and IL-6. Consistently, in mice, topical applications of the TLR7/TLR8 ligand imiquimod induce psoriasis-like skin inflammation. KCs can be viewed as an integral part of the skin-resident immune system, because they may act as APCs, produce innate immune mediators, and contribute to the skin homing and local activation of immune cells. KCs express TLRs and respond to microbial stimuli by producing large amounts of cytokines (e.g. TNF-α, IL-1α, IL-6 and IL-18), chemotactic chemokines [e.g. IL-8 and CCL20 (CC chemokine ligand 20)] and antimicrobial peptides [e.g. HBD (human β-defensin)-2, HBD-3 and LL37]. Moreover, the psoriatic plaque is characterized by a marked infiltration of activated CD4+ and CD8+ T-cells. CD4+ T-cells infiltrate mainly the dermis, whereas CD8+ T-cells are present in the epidermis. Th (helper) 1 and Th2 are the best understood effector CD4+ T-cells formed during immune responses. Th1 cells produce IFN-γ and TNF-α, and mediate immune responses against intracellular bacteria, viruses and tumour cells. Th2 cells make mostly IL-4, IL-5 and IL-13, and stimulate humoral responses against extracellular parasites. Another subset of Th cells, termed Th17 cells, which are predominantly CD161-expressing cells, secrete IL-

17A and IL-17F, and are involved in the activation of neutrophils and immunity to bacteria and fungi. TGF-β1 (transforming growth factor-β1) concentration and the concomitant presence of at least one proinflammatory cytokine are key factors in human Th17 differentiation. These cells, together with Th1 cells, (121), appear to lie at the very heart of the pathogenesis of psoriasis (119). Indeed, IL-17 produced by Th17 cells was shown to promote the production of IL-6, IL-8, GM-CSF (granulocyte/macrophage colony-stimulating factor) and ICAM-1 (intercellular adhesion molecule-1) in KCs, synergizing with IFN-γ. They can also produce IL-21, IL-22 and IL-26. IL-21 acts on Th17 cells to amplify its own synthesis. IL-21 also up-regulates IL-23R expression on T-cells, thus making Th17 cells responsive to IL-23. Altogether, these events generate a positive-feedback loop that helps amplify the Th17 lineage. More recently, it has been shown that the IL-22 expression profile may differ from that of IL-17A, and that IL-22-producing T-cells, termed Th22 cells, could represent a T-cell subset that is distinct from typical Th17 cells (122) Traditionally, psoriasis has been classified as a Th1-associated disease, because T-cells infiltrating the lesional skin of psoriatic patients produce high levels of IFN-γ. IL-12, the major Th1-inducing factor in humans, is also highly expressed (Figure1). However, in the psoriatic plaque, there is also elevated synthesis of Th17-related cytokines, such as IL-17A, IL-17F, IL-21 and IL-22 (Figure 1), as well as enhanced production of IL-23, a heterodimeric cytokine composed of IL-23p19 and IL-12p40 subunits which amplifies Th17 cell responses and causes psoriasis lesions when administered intradermally to mice. A functional role of Th17 cells in psoriasis is suggested by the demonstration that both IL-21 and IL-22 can induce keratinocyte hyperplasia and that Th17 cytokine levels decrease during successful anti-TNF-α treatment. IL-22 also triggers the production of antimicrobial peptides and expression of genes involved in epidermal differentiation and survival. Studies in mice have shown that IL-22 induces keratinocyte hyperplasia and acanthosis, and that some biological effects of

IL-22 are amplified by TNF-α, as a result of the ability of TNF-α to enhance IL-22 receptor expression (122). Both Th1 and Th17 cytokines induce keratinocytes to produce CCL20, a chemoattractant for CCR6-expressing DCs and T-cells, thus providing a positive feedback loop that sustains the accumulation of these cells in the psoriatic skin. IL-17A and IL-22 are powerful inducers of IL-20 by DCs and proliferating keratinocytes. IL-20 is highly produced in psoriasis, and its overexpression in transgenic mice causes epidermal thickening. Interestingly, the skin alterations in IL-20-transgenic mice occur without immune cell infiltration, suggesting that IL-20 is a downstream mediator in the psoriasis-associated immuno-inflammatory cascade. Therefore the IL-23/Th17 pathway appears to be central in the pathogenesis of psoriasis, orchestrating both the induction and maintenance of skin inflammatory response by regulating the secretion of inflammatory cytokines and chemoattractants, and the proliferative response of psoriatic KCs through the production of mitogenic cytokines (IL-22 and IL-21).



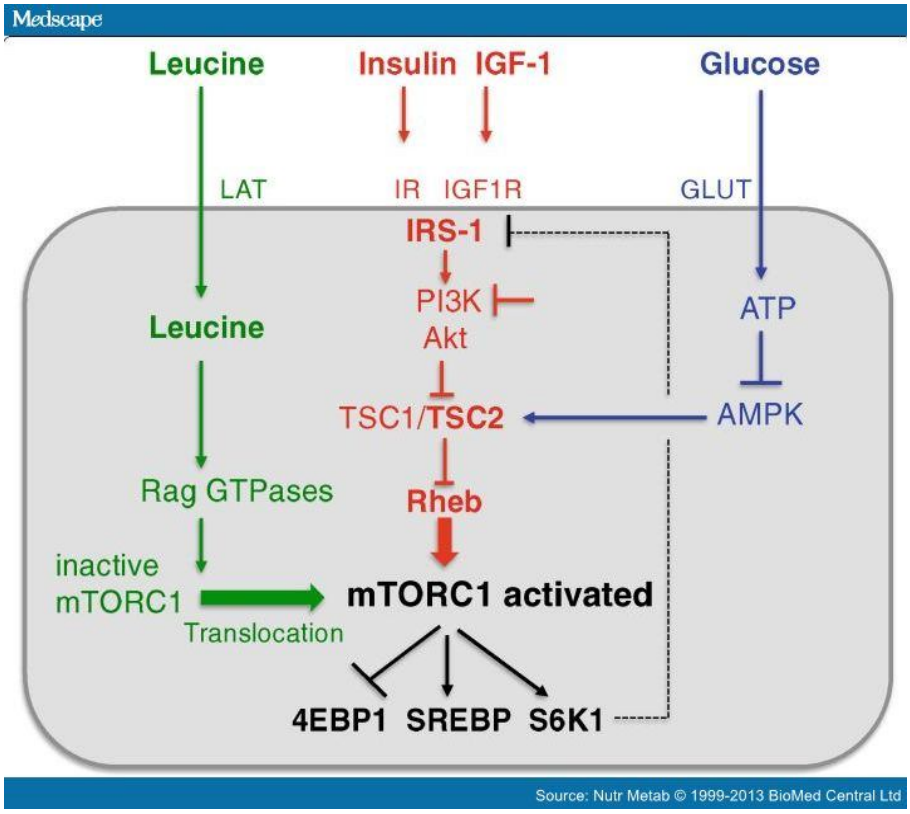
Pathogenesis of acne

Acne is an inflammatory process involving the pilosebaceous unit-hair follicles in the skin with a prevalence rates of over 85% in adolescents (111). The clinical features of acne include seborrhoea (excess grease), non-inflammatory lesions (open and closed comedones), inflammatory lesions (papules and pustules), and various degrees of scarring. The distribution of acne corresponds to the highest density of pilosebaceous units (face, neck, upper chest, shoulders, and back). Nodules and cysts comprise severe nodulocystic acne.

Four processes have a pivotal role in the formation of acne lesions: inflammatory mediators released into the skin; alteration of the keratinisation process leading to comedones; increased and altered sebum production under androgen control (or increased androgen receptor sensitivity) and follicular colonisation by *P acnes.* (123). The exact sequence of events and how they and other factors interact remains unclear.

Inflammatory acne process might involve CD4+ lymphocytes and macrophages (124). Defective terminal keratinocyte differentiation leads to comedo formation under the influence of androgens and qualitative changes in the sebum lipids that induce IL-1 secretion (125). Sebaceous glands are an important part of the innate immune system, producing a variety of antimicrobial peptides, neuropeptides, and antibacterial lipids such as sapienic acid. Each sebaceous gland functions like an independent endocrine organ influenced by corticotrophin-releasing hormone, which might mediate the link between stress and acne exacerbations (126). Vitamin D also regulates sebum production, and IGF-1 might increase sebum through sterol-response element-binding proteins (SREBs) (127). Matrix metalloproteinases in sebum have an important role in inflammation, cell proliferation, degradation of the dermal matrix, and treatment responsiveness (128). Sebaceous follicles containing a microcomedone provide an anaerobic and lipid-rich

environment in which *P acnes* flourishes (129). Lipogenesis is directly augmented by *P acnes* (130). Colonization of facial follicles with *P acnes* follows the asynchronous initiation of sebum production which might explain why treatment with isotretinoin too early can need to be followed up with subsequent courses, as new previously *P acnes*-naive follicles become colonized and inflamed. Unique *P acnes* strains with different bacterial resistance profiles colonize different pilosebaceous units and induce inflammation by the activation of toll-like receptors in keratinocytes and macrophages (131) In-vitro work suggests that *P acnes* could behave like a biofilm within follicles, leading to decreased response to antimicrobial agents (132). *P acnes* resistance to commonly used oral antibiotics for acne affects treatment response, suggesting that direct antimicrobial effects might be important in addition to the anti-inflammatory actions of antibiotics.(133).



Chapter III

Experimental Design

Objective:

Based on the association of mTOR with inflammatory conditions, we sought to investigate its possible involvement in cutaneous inflammation. Indeed, although the current interest in the development of mTOR inhibitors is focused almost exclusively on their potential as anticancer agents, a better understanding of mTOR biology/complex signalling might lead to new therapeutic employments, also in inflammatory diseases.

Materials and methods

Human subjects

The overall study population included: 15 patients affected by moderate-to-severe psoriasis, 5 patients with allergic contact dermatitis (ACD), 5 patients with atopic dermatitis (AD), 3 patients affected by colon cancer with hepatic and lymph nodes metastases presenting a skin inflammatory rash induced by cetuximab, an epidermal growth factor receptor (EGFR) inhibitor, 5 patients with acne and 10 healthy controls. All the patients were i) in a similar age range ii) not affected by other comorbidities and iii) in 1-month wash out period from systemic treatment. 3 mm skin biopsies were performed to all patients in the skin affected by the dermatosis and in uninvolved, clinically normal skin. In addition, skin specimens (3 mm Φ) were taken from psoriatic plaques of 5 patients before (T0) and after 16 weeks (T1) of treatment with adalimumab, an inhibitor of tumor necrosis factor-α (TNF-α) (administered subcutaneously 80 mg at week 0 and successively 40mg eow). Healthy skin specimens, obtained from 10 healthy donors, who had undergone plastic surgery, were used as controls. Blood samples were obtained from

5 healthy volunteers as well as 5 patients with psoriasis for the isolation of peripheral blood mononuclear cells (PBMC). The experimental protocol was approved by the Ethics Committee of our institution and was conformed to the principles outlined in the Declaration of Helsinki. Each subject gave written informed consent before entering the study.

Purification of PBMC

PBMC were separated by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Total RNA was isolated by RNeasy Mini Protocol (Qiagen, Valencia, CA, U.S.A.) and cDNA was prepared (Transcriptor High Fidelity cDNA Synthesis; Roche, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions.

Ex vivo full-thickness skin organ culture

Skin biopsies (3 mm diameter and length) of 10 healthy subjects undergoing to plastic surgery were cultured as follows: a hole was punched in a transwell filter (pore size 1 μm; Beckton Dickinson Labware, Franklin Lakes, NJ); the biopsy was placed in a 12-well culture plate (Beckton Dickinson Labware) containing 1 ml medium with the epidermis facing upwards at the liquid-air interface and the dermis suspended in the culture medium. Biopsies were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) containing 10% FBS (GIBCO), 2 mM L-glutamine (GIBCO) and antibiotics (100 IU/ml penicillin G, 100 μg/ml streptomycin, GIBCO) under normal conditions (atmosphere containing 5% CO2 at 37°C). Incubation with TNF-α (GIBCO) and IL-17A (Biosource International, Camarillo, CA) was performed at 20 ng/ml and 10 ng/ml, respectively, for 24 h.

Cell cultures

HaCaT cells were grown in DMEM, GIBCO, containing 10% fetal bovine serum (FBS, GIBCO), 2 mM L-glutamine (GIBCO) and antibiotics (100 IU/ml penicillin G, 100 µg/ml streptomycin, GIBCO). Human epidermal keratinocytes (KCs) were isolated from normal healthy skin of surgical remnants. Single-cell suspensions of KCs were prepared using standard methods (19). KCs used for experiments were in the second or third passage. Both cells were cultured at 37°C with 5% CO2 in air. For in vitro stimulation assays, HaCaT cells and primary KCs, at 70–80% confluence, were stimulated with TNF-α (20 ng/ml GIBCO), IL-17A (10 ng/ml Biosource International, Camarillo, CA), or their combination for 24h. HaCaT cells were also irradiated with 100mJ/cm² of ultraviolet (UV)B (TL12 lamp Philips, Eindhoven, The Netherlands) and cultured for 24h.

RNA extraction, cDNA synthesis and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was extracted (RNeasy Mini Protocol Qiagen, Valencia, CA) from skin biopsies as well as cell cultures and cDNA was prepared (Transcriptor High Fidelity cDNA Synthesis, Roche, Indianapolis, IN) according to the manufacturer's instructions. qRT-PCR (LightCycler, Roche) was used to analyze levels of expression of mTOR and its major up-stream as well as down-stream regulators such as tuberous sclerosis protein (TSC) 1 and 2, Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and Ribosomal protein S6 kinase beta-1 (S6K1). Relative mRNA levels were determined by the comparative threshold cycle method, and their expression was normalized to the expression of 18S mRNA. PCR primers (mTOR, TSC1, TSC2, 4EBP1, S6K1, 18S) were designed based on published sequences, and their specificity was verified with BLAST alignment search. To confirm amplification of the expected size fragment, amplification

products were characterized by agarose gel electrophoresis. Melting curve analysis was carried out after completion to confirm the presence of single amplified species.

Immunohistochemistry

The immunohistochemical detection of mTOR was carried out on lesional and non lesional skin samples of 5 psoriatic and 5 acneic patients. Healthy skin samples were used as controls. Skin samples were immediately placed in tissue freezing medium (Jung, Leica, Wetzlar, Germany) and stored at −80° C. Five micrometer sections were cut with a cryostat and fixed with cold methanol for 10 min. The Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was used as follows: sections were incubated with blocking solution (horse serum diluted in buffer: phosphate buffered saline [PBS] + bovine serum albumin 1%) for 20 min at 22° C. Biopsies were stained with rabbit anti-human mTOR (2.5 µg/ml LS-B650 IHC-plus LifeSpan BioScieces) and incubated overnight at 4° C. The sections were then washed in buffer and incubated with biotinylated secondary antibody for 30 min at room temperature. Peroxydase activity was revealed using DAB substrate (ImmPACT DAB, Burlingame, CA). Counterstaining was performed with hematoxylin.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc, La Jolla, CA). Student's t test or ANOVA (when comparing more than 2 groups) were used to calculate statistical differences. Values of $p < 0.05$ were considered significant.

Results

mTOR gene expression is increased in skin inflammatory diseases

mTOR gene expression was significantly increased in psoriasis, ACD, AD as well as in EGFR inhibitor induced cutaneous rash, compared to healthy skin (Fig. 1A). Psoriasis mTOR expression was also enhanced in non-lesional skin of psoriatic patients respect to healthy skin (Fig. 1B). Conversely, mTOR gene expression resulted decreased in PBMC isolated from psoriatic subjects when compared to healthy controls (Fig. 1C).

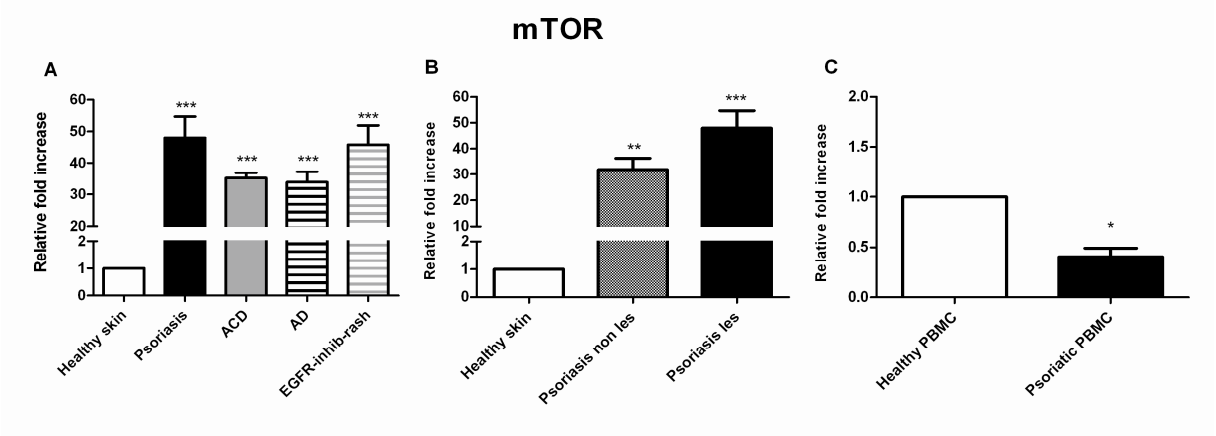


Fig. 1. *mTOR gene expression in inflammatory skin diseases: A) psoriasis, ACD, AD and EGFR-inhibitor-induced cutaneous rash; B) psoriatic lesional and non lesional skin.* Data were compared with healthy skin, normalized to the housekeeping gene 18S and expressed as mean ± SD. *C) healthy and psoriatic PBMC.* Data were compared with healthy PBMC, normalized to the housekeeping gene 18S and expressed as mean ± SD. ACD=allergic contact dermatitis, AD=atopic dermatitis, EGFR=epithelial growth factor receptor, les=lesional, PBMC=peripheral blood mononuclear cells, SD=standard deviation, *p<0.05, **p<0.01, ***p<0.001

mTOR up-stream regulators and down-stream effectors in skin inflammatory diseases

To evaluate the effective enhancement of mTOR in these skin inflammatory diseases we also investigated the expression of mRNA levels of key negative upstream regulators of

mTOR such as TSC1 and TSC2. TSC1 and TSC2 gene expression were not enhanced in psoriasis and ACD and were significantly reduced in AD compared to healthy skin (Fig. 2A-B); particularly, as regards EGFR inhibitor induced cutaneous rash, TSC1 levels were not significantly increased, whereas TSC2 gene expression was reduced.

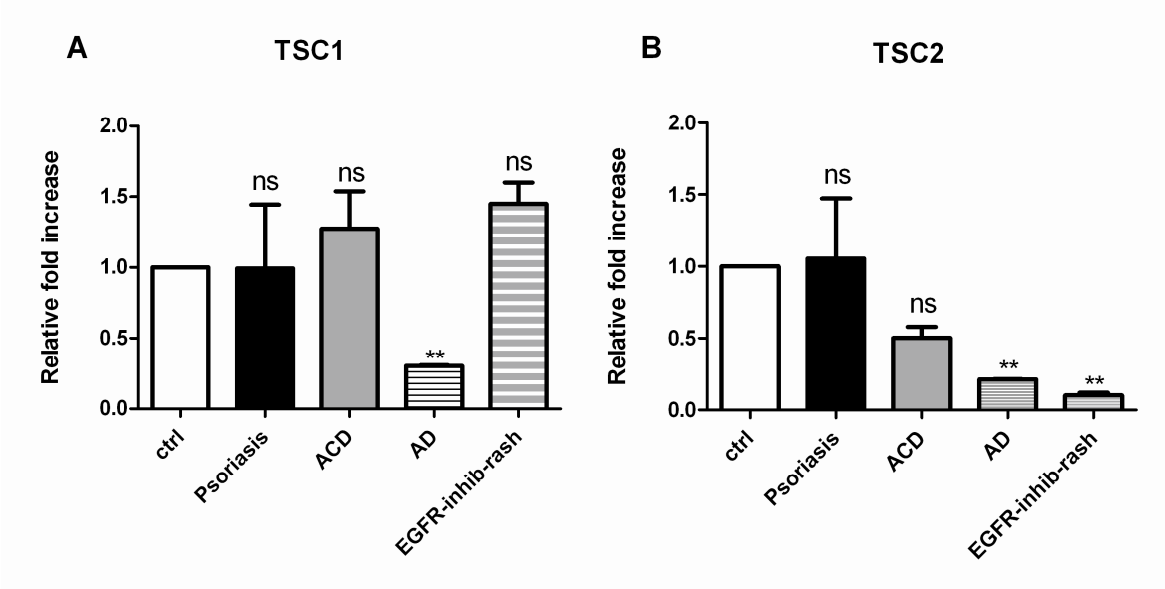


Fig. 2. *A) TSC1 and B) TSC2 gene expression in psoriasis, ACD, AD and EGFR-inhibitor-induced cutaneous rash.* Data were compared with healthy skin, normalized to the housekeeping gene 18S and expressed as mean ± SD. ACD=allergic contact dermatitis, AD=atopic dermatitis, EGFR=epithelial growth factor receptor, SD=standard deviation, TSC1=tuberous sclerosis protein 1, TSC2=tuberous sclerosis protein 2, *p<0.05, **p<0.01, ***p<0.001

Since our experiments showed that mTOR may be involved in skin inflammation we have also investigated gene expression of its major downstream effectors such as 4EBP1 and S6K1, which are known as the best output of mTORC1. Neither 4EBP1 nor S6K1 were significantly augmented in psoriasis, AD, ACD or EGFR inhibitor induced cutaneous rash (Fig. 3A-B).

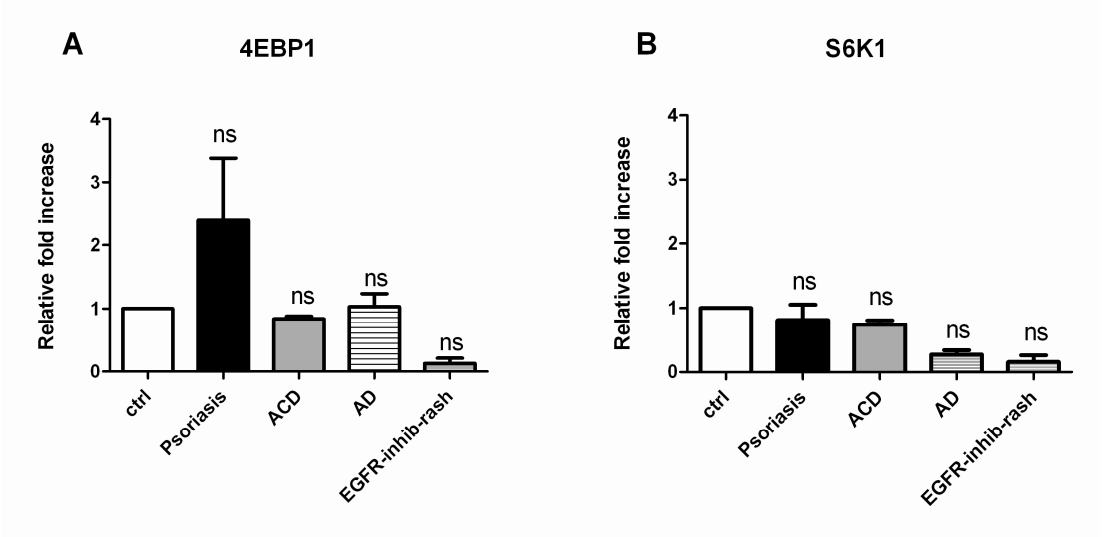


Fig. 3. *A) 4EBP1 and B) S6K gene expression in psoriasis, ACD, AD and EGFR-inhibitor-induced cutaneous rash.* Data were compared with healthy skin, normalized to the housekeeping gene 18S and expressed as mean ± SD. ACD=allergic contact dermatitis, AD=atopic dermatitis, 4EBP1= Eukaryotic translation initiation factor 4E-binding protein 1, EGFR=epithelial growth factor receptor, SD=standard deviation, S6K= Ribosomal protein S6 kinase beta-1 (S6K), *p<0.05, **p<0.01, ***p<0.001

mTOR is strongly expressed in psoriasis (both lesional and non-lesional skin)

As an additional confirmation, immunohistochemical studies demonstrated that mTOR was effectively over-expressed in skin inflammatory diseases. In particular, as regards psoriasis, mTOR kinase was equally strong expressed through all epidermal layers in lesional (Fig. 4A) as well as non-lesional psoriatic skin (Fig. 4B); but only weakly expressed in the epidermis from healthy individuals (Fig. 4C).

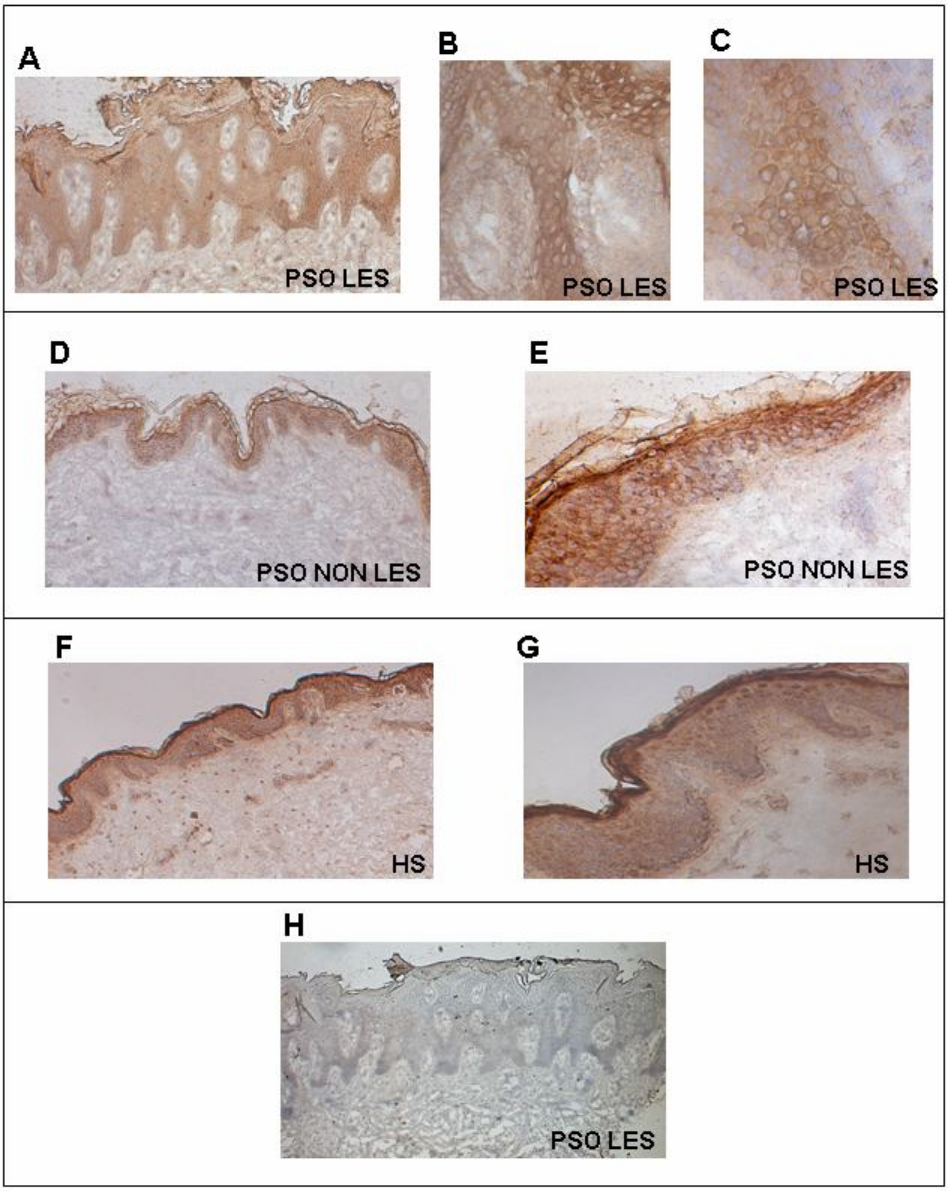


Fig. 4. *Immunohistochemical detection of mTOR in A-B-C) lesional psoriatic skin; D-E) non lesional psoriatic skin and F-G) healthy skin; F) negative control.* DAB staining X6 (A, D, F, H), X25 (B, E, G), X40 (C). Each picture is representative of data from 5 different patients affected by moderate-to-severe psoriasis or healthy subjects. PSO LES=psoriatic lesional skin; PSO NON LES=psoriatic non lesional skin; HS=healthy skin

mTOR gene expression is increased by UVB irradiation, but not by TNF-α and IL-17A

Given that mTOR was enhanced in skin inflammatory diseases, we sought to analyze if a pro-inflammatory setting was determinant for mTOR induction in KCs as well as skin organ culture. More specifically, we investigated whether UVB irradiation or psoriasis-associated Th1 and Th17 cytokines could interfere with mTOR gene expression. Our results showed that IL-17A, TNF-α or their combination, were not able to increase mTOR, neither in HaCaT nor in KCs cultures and in *ex vivo* full-thickness skin organ culture (Fig. 5A-C). Interestingly, 100mJ/cm² UVB irradiation was able to slightly increase mTOR gene expression in HaCaT cells (Fig. 5A).

mTOR gene expression in psoriasis is not altered by anti-TNF-α treatment

Since mTOR was enhanced in psoriasis plaques, we sought to investigate if a proper systemic treatment was able to down-regulate it. More specifically, we assessed mTOR gene expression in lesional psoriatic skin of 5 patients before and after 16 weeks of anti-TNF-α treatment. Although clinical improvement was assessed in all patients, with 60% (n=3) of them reaching PASI75 (75% improvement of PASI score) and 40% (n=2) reaching PASI50 (50% improvement of PASI score), mTOR gene expression was not altered by anti-TNF-α treatment (Fig. 5D).

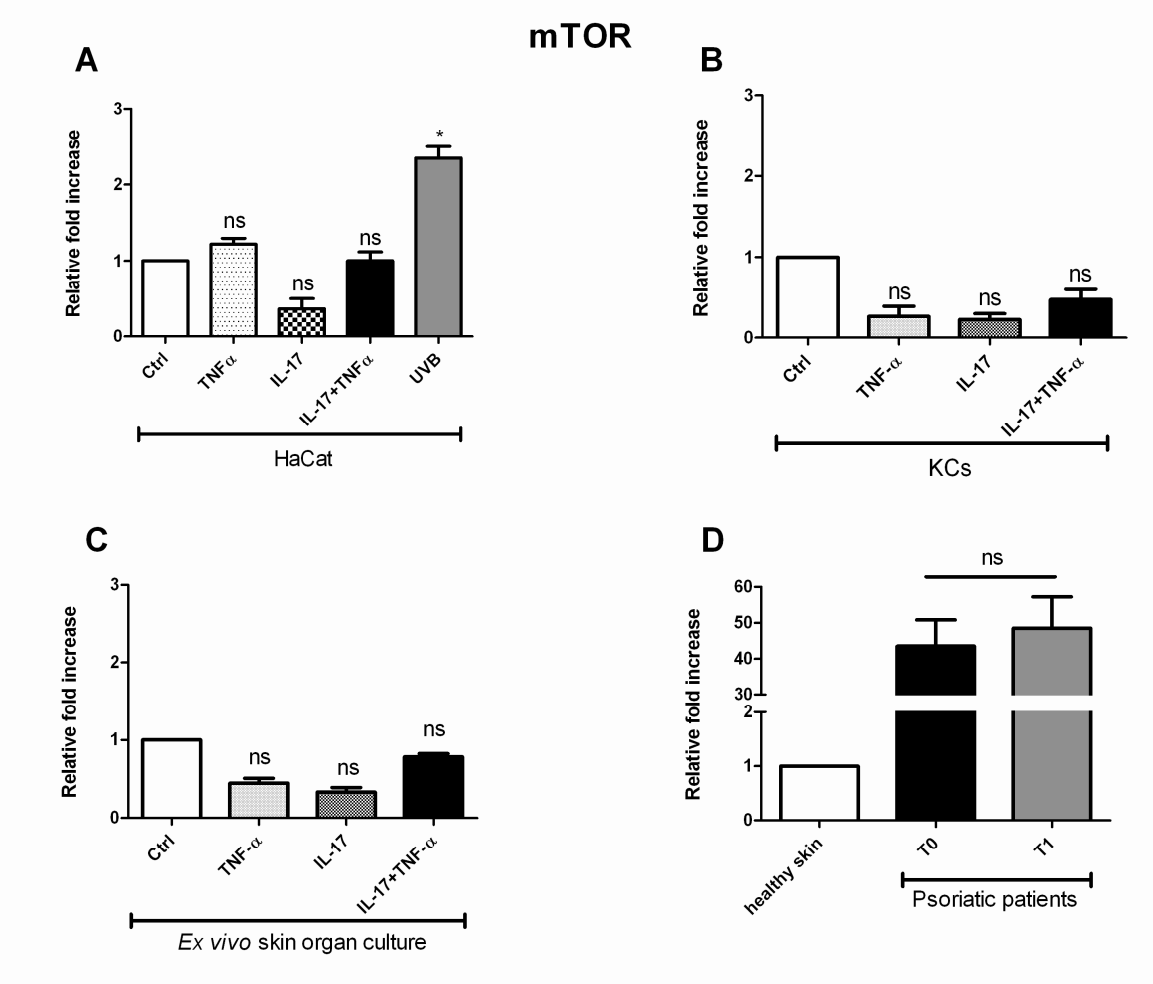


Fig. 5. *mTOR gene expression in: A) HaCaT cells, B) primary KCs, C) ex vivo full-thickness skin organ cultures and D) 5 psoriatic patients before (T0) and after (T1) 16 weeks of treatment with adalimumab;* A) UVB dose used: 100 mJ/cm²; A-B-C) stimulations with pro-inflammatory *stimuli* TNF-α (20 ng/ml), IL-17A (10 ng/ml), and TNF-α + IL-17A were performed for 24h. Data were compared with untreated HaCaT cells, primary KCs, *ex vivo* skin organ culture and healthy skin, respectively; normalization to the housekeeping gene 18S was performed and displayed as mean ± SD of three independent experiments. Ctrl=control, KCs=keratinocytes, TNF-α=tumor necrosis factor-α, **p*<0.05, ***p*<0.01, ****p*<0.001.

mTOR expression is modulated in acne

Recently, mTOR has emerged having a role in acne pathogenesis, but very little is known. For this reason we extend our study analyzing gene profile expression of mTOR, its upstream regulators (TSC1 and 2) and its downstream effectors (4EBP1 and S6K1) in lesional and non-lesional skin biopsies of patients with acne. mTOR gene expression was significantly increased in acne, both in non-lesional and lesional skin respect to healthy controls, as well as IL-8 (Fig. 6A). As an additional confirmation, immunohistochemical studies demonstrated that mTOR was effectively over-expressed. In particular, mTOR kinase was equally strong expressed through all epidermal layers in lesional as well as non-lesional acneic skin; but only weakly expressed in the epidermis from healthy individuals (data not shown).

Conversely, mRNA levels of TSC1 and TSC2, 4EBP1 and S6K1 were not enhanced in acne.

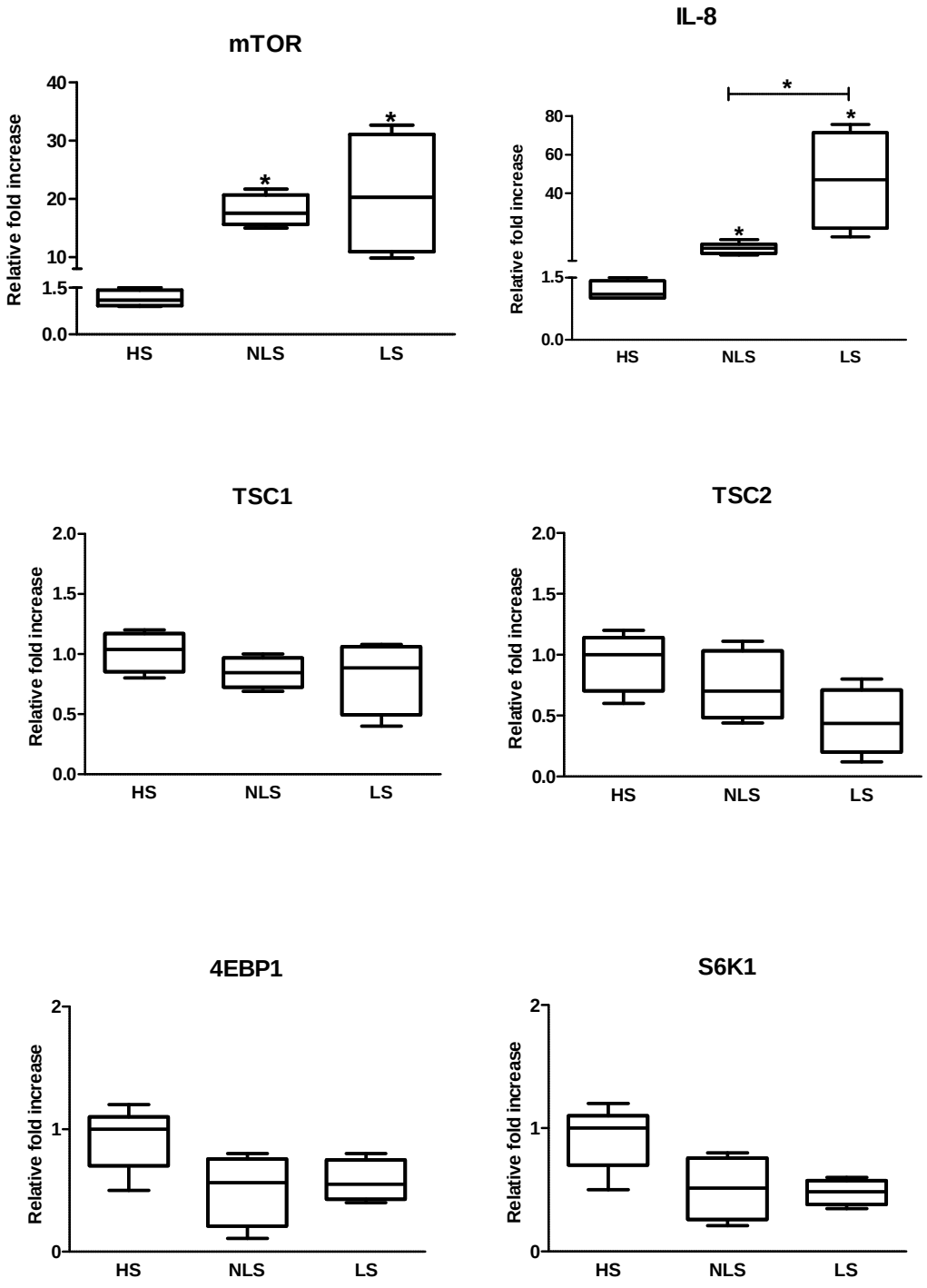


Fig. 6 mTOR, IL-8, TSC1 ,TSC2, 4EBP1 and S6K1 gene expression in acneic non lesional and lesional skin. Data were compared with healthy skin, normalized to the housekeeping gene 18S and expressed as mean ± SD. 4EBP1= Eukaryotic translation initiation factor 4E-binding protein 1 S6K= Ribosomal protein S6 kinase beta-1, SD=standard deviation, (S6K), TSC1=tuberous sclerosis protein 1, TSC2=tuberous sclerosis protein 2, *p<0.05, **p<0.01, ***p<0.001

Discussion

Although numerous literature evidences suggest a pro-inflammatory role for mTOR (12-15, 106-109), little is known about its relationships with skin inflammation. We investigated whether mTOR was involved in some of the most common skin inflammatory diseases, evaluating its mRNA levels in psoriasis, ACD and AD. Our results showed that mTOR gene expression was significantly increased in these dermatoses. Moreover, the evaluation of mTOR gene expression in EGFR-inhibitor-induced skin reaction confirmed the modulation of mTOR pathways as a constant marker of skin inflammatory processes. However, mTOR, a serine/threonine kinase, functions as a central node in a complex network of phosphorylation/dephosphorylation signaling pathways that activate the translational machinery and promote cell growth. For this reason, the enhancement of mTOR previously found do not explain its activation in the investigated skin inflammatory diseases thought a traditional post-translational pathway. These results were confirmed and validated by the investigation of the expression of the major negative up-stream regulators of mTOR such as TSC1 and 2. Indeed, it is well known that TSC1 and 2 negatively regulates the kinase activity of mTORC1 by converting a GTP-binding protein, Ras omolog enriched in brain (Rheb), into its inactive GDP-bound state (134). TSC1 and TSC2 gene expression was not increased in psoriasis and ACD and significantly reduced in AD compared to healthy skin; as regards EGFR inhibitor induced cutaneous rash, TSC1 levels were not significantly increased, whereas TSC2 gene expression was reduced. Therefore, these gene expression data did not provide evidence that Akt activates mTOR, at least in part, through the phosphorylation and inactivation of TSC1/2 heterodimer. Further evidences about mTOR over-expression in skin inflammatory diseases were powered by immunohistochemical studies on skin biopsies of lesional and non lesional psoriatic skin. A strong expression of mTOR was found through all

epidermal layers in both lesional and non lesional psoriatic skin compared to the epidermis from healthy individuals. Particularly concerning psoriasis, our data are in line with those reported by Buerger et al. who recently showed for the first time an increase in mTOR expression and phosphorylation at Ser2448 (a site representative for mTOR activation) in lesional and non-lesional psoriatic skin compared to healthy skin through immunoistochemical and immunofluorescent studies, respectively (135). On the other hand, mTOR mRNA level was decreased in PBMC of psoriatic patients, suggesting that mTOR signalling might be specifically involved in skin inflammation rather than in a systemic lymphocytes activation. All these data underline the possible involvement of mTOR in cutaneous inflammatory diseases. It is reported that mTOR is implicated in the development of several inflammatory and metabolic disorders, such as diabetes, metabolic syndrome, rheumatoid arthritis and Crohn's disease (136-141). Chong *et al.* reported that mTOR can alter insulin signalling leading to insulin resistance in the cardiovascular system during diabetes mellitus (136), whereas Dazer *et al.* underlined that alterations of mTOR signalling pathway, especially regarding energy homeostasis and metabolism, are implicated in metabolic syndrome, which is strictly correlated to diabetes (12). Furthermore, mTOR represents an important linkage between synovitis and structural damage in rheumatoid arthritis and its inhibition by rapamycin or everolimus is able to reduce synovial osteoclast formation, protecting against local bone erosions and cartilage loss (13). In addition, mTOR has been reported to be related to the triggering of Crohn's disease; Indeed, mTOR plays a major role in the regulation of autophagy (137) whose impairment represents a critical initiating point in this disease pathogenesis (138, 139). Moreover, inhibitors of mTOR are reported to be useful in treating Crohn's disease, as highlighted by several case reports (140, 141). To gain additional information about the involvement of mTOR in the pathogenesis of skin inflammatory diseases we also evaluated the gene expression of its main down-stream outputs such as 4EBP1 and S6K1. Nor 4EPB1 neither S6K1 were significantly enhanced in

psoriasis, ACD, AD as well as EGFR inhibitor induced cutaneous rash. Therefore, it seems that mTOR pathway activated in skin inflammatory diseases may be different from the traditional one implicating 4EBP1 and/or S6K1. However, these data were not sufficient for arising such conclusion because mTOR regulates protein synthesis mainly through the phosphorylation and inactivation of 4EBP1 and through the phosphorylation and activation of S6K1. Indeed, very recently Buerger *et al.* (135) showed that S6K1 is activated in psoriasis lesions compared to non-lesional skin as well as one of its major target, the ribosomal protein S6. Nevertheless, the authors analyzed only a very small group of subjects (3 psoriatic patients); furthermore, the activation of S6K1 was found in only 2 out of 3 psoriatic patients. Therefore, the pathway triggered by mTOR in psoriasis and consequently in skin inflammation is still not so clear; more studies and researches are needed to clarify its potential involvement in the disease.

Hence, to further analyze the role of mTOR in skin inflammation, we reproduced a psoriasis-like pro-inflammatory milieu *in vitro*, stimulating KCs as well as skin organ culture with TNF-α, IL-17A and their combination; No alterations of mTOR gene expression were encountered. Young et al. (142) showed that rapamycin reduced TNF-α expression in KCs, and so hypothesized that, TNF-α could be able to enhance mTOR, being so implicated in cutaneous inflammation. Conversely, Williamson et al. reported that elevated concentrations of TNF-α in circulating blood or in cell culture medium of C2C12 myotubes inhibit insulin-mTOR-stimulated pathways (143).

Furthermore, Di-Lin *et al.* showed that mTOR inhibits the anti-inflammatory action of Liver X Receptor (LXR-α) and Pregnane X Receptor (PXR) in TNF-α-mediated responses, suggesting that it may act as a negative regulator of lipid homeostatsis (145).

We found that UVB irradiation did enhance mTOR in the 3 analyzed models. The mechanisms by which UV modulates inflammation, in the immunoactivation-immunosuppression balance, is complex and not completely understood, but it is well know

its ability in increasing multiple pro-inflammatory cytokines. Our data are in line with previous studies which reported that UVB induced mTOR activation in HaCat cells (146) and primary KCs (147). On the light of these *in vitro* results, we decided to assess whether mTOR expression was influenced by anti TNF-α therapy. Interestingly adalimumab treatment did not reduce mTOR mRNA expression, although all patients reported clinical improvement. All together, these results suggested that mTOR is involved in cutaneous inflammatory process, but through a signalling not directly dependent from Th1-Th17 pathway.

Recently, mTOR has emerged having a role in acne pathogenesis, but very little is known. For this reason we extend our study analyzing gene profile expression of mTOR, its upstream regulators (TSC1 and 2) and its downstream effectors (4EBP1 and S6K1) in lesional and non-lesional skin biopsies of patients with acne. However, the not increased mRNA levels of mTOR downstream effectors 4EBP1 and S6K1 found in our experiments do not imply that mTORC1 is not activated in the inflammatory mechanism of acne. Indeed, placebo-controlled studies have demonstrated that high glycaemic load diets aggravate acne, result in postprandial hyperinsulinaemia and increase serum levels of free IGF-1 (112-114). Epidemiological as well as clinical evidence confirmed that milk and other insulinotropic dairy products induce or aggravate acne (114) because milk functions as an endocrine growth–promoting signalling system of mammals, which activates mTORC1 signalling but inhibits FoxO1-dependent gene regulation (110). FoxO1 is an important transcription factor that, like mTORC1, modulates the expression of genes involved in the regulation of cell proliferation, apoptosis, anti-oxidative stress responses and regulation of glucose and lipid metabolism. In particular, increased insulin/IGF-1 signaling and activation of the PI3K/Akt-pathway results in Akt-mediated nuclear phosphorylation of FoxO1 protein, thereby promoting its extrusion from the nucleus into the cytoplasm where it is inhibited. This shuttling mechanism functions as a molecular switch for FoxO1-mediated gene regulation.

Moreover, FoxO1 not only suppresses protein synthesis and cell growth, but also lipid metabolism because regulates the key transcription factor of lipid synthesis, SREBP (116). IGF-1, through mTORC1 activation, induced SREBP expression and enhanced lipogenesis in sebocytes via activation of the PI3K/Akt pathway, whereas FoxO1 antagonized the expression of SREBP.

Conclusions

In conclusion, our study provides evidence that mTOR is involved in cutaneous inflammatory process but deeper insights into the comprehension of its molecular pathway may help to understand the effective role of mTOR in the pathogenesis and treatment of diseases such as psoriasis and acne. Specially, our findings highlight the necessity of investigating activated mTOR protein as well as its upstream regulators and downstream effectors to better penetrate the complex biology of mTOR.

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