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IDENTIFICATION OF NEGATIVE REGULATORS OF THE *CUTIBACTERIUM ACNES*-INDUCED INNATE IMMUNE ACTIVATION IN HUMAN EPIDERMAL KERATINOCYTES

Ph.D. thesis

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Table of Contents

1 Introduction 8

1.1 Skin anatomy and function 8

1.2 Localization and composition of the cutaneous microbiota 9

1.3 The role of cutaneous microbiota in healthy skin and the pathogenesis of different skin diseases 9

1.4 Skin microbiota-induced innate immune activation in keratinocytes 10

1.4.1 *C. acnes* recognition by human epidermal keratinocytes and their role in acne pathogenesis 11

1.5 Factors contributing to the negative regulation of TLR signaling pathways 13

1.5.1 SIGIRR, TOLLIP, TNFAIP3 and TNIP1 13

2 Aims 16

3 Materials and Method 17

3.1 Cell cultures and models 17

3.2 Treatments 17

3.3 Transfection, plasmids and siRNA-mediated gene silencing 18

3.4 ELISA 19

3.5 RNA isolation, cDNA synthesis and real-time RT-PCR 19

3.6 Protein isolation and western blot analysis 20

3.7 Fluorescence microscopic analysis 20

3.8 Data analysis 21

3.9 Statistical analysis 21

4 Results 22

4.1 Studying the expression levels and the possible role of SIGIRR in the *C. acnes*-induced innate immune activation of keratinocytes 22

4.2 Studying the expression levels and the possible roles of TOLLIP, in the *C. acnes*-induced innate immune activation of keratinocytes 24

4.3 Studying the expression levels and the possible role of TNFAIP3 in the *C. acnes*-induced innate immune activation of keratinocytes 26

4.3.1 *C. acnes* bacterium increases TNFAIP3 expression in keratinocytes 27

4.3.2 Changes in *C. acnes*-induced TNFAIP3 expression are dependent on JNK and NF-κB signaling pathways 29

4.3.3 TNFAIP3 affects the basal and *C. acnes*-induced levels of inflammatory mediators in keratinocytes 30

4.3.4 TNFAIP3 expression differs in acne lesions 32

4.4 TNIP1 is expressed in keratinocytes and its expression increases in the presence of *C. acnes* 32

4.4.1 *C. acnes*-induced expression changes of TNIP1 are regulated by different signaling pathways in keratinocytes 34

4.4.2 Regulation of TNIP1 levels in response to *C. acnes* bacterium is not specific for HPV-KER cells 34

4.4.3 TNIP1 down-regulates both constitutive and *C. acnes*-induced inflammatory cytokines and chemokines expression 35

4.4.4 ATRA induces TNIP1 expression and affects the levels of downstream targets of the TLR signaling pathway 38

5 Discussion 40

6 Summary 49

7 Acknowledgement 50

8 References 51

Abbreviation

AB/AM	antibiotic/antimycotic
AD	atopic dermatitis
AMP	antimicrobial peptide
AP-1	activator protein 1
ATRA	all-trans retinoic acid
C. acnes	*Cutibacterium acnes*
C/EBPB	CCAAT enhancer binding protein beta
CCL5	C-C motif chemokine ligand 5
ChIP	chromatin immunoprecipitation
CREB	cAMP response element-binding protein
DAPI	4', 6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DRE	downstream-responsive element
DREAM complex	consisting of E2F dimerization partner (DP), retinoblastoma (RB), E2F proteins, and the multivulva class B (MuvB) complex
ELISA	enzyme linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinases 1 and 2
FADD	Fas associated via death domain
GSK3	Glycogen synthase kinase 3
hBDs	human β defensins
HLMVEC	human lung microvessel endothelial cells
HPV-KER	Human Papillomavirus immortalized keratinocyte cell line
IKKi	inhibitor of nuclear factor kappa B kinase subunit epsilon
IKKα	inhibitor of nuclear factor kappa B kinase subunit alpha
IKKβ	inhibitor of nuclear factor kappa B kinase subunit beta
IL	interleukin
IL1RL1	interleukin 1 receptor like 1
IRAK	interleukin 1 receptor associated kinase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharides
MAPKs	mitogen-activated protein kinases
MOI	multiplicity of infection
MYD88	MYD88 innate immune signal transduction adaptor
NEMO	inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (IKBKG)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK	normal human epidermal keratinocyte
OS	*ex vivo* organotypic skin

p105	nuclear factor kappa B subunit 1 and 2
p38	p38 mitogen-activated protein kinases
PAMPs and DAMPs	pattern- and damage-associated molecular patterns
PBS	phosphate-buffered saline
PSO	psoriasis
RARE	retinoic acid response element
RARs	retinoic acid receptors
RIP1	receptor interacting protein 1
S. epidermidis	*Staphylococcus epidermidis*
S. aureus	*Staphylococcus aureus*
SCFA	short chain fatty acid
SD	standard deviation
SE	standard error
SIGIRR	single Ig and TIR domain containing
SNP	single nucleotide polymorphisms
SOCS1	suppressor of cytokine signaling 1
SP1	Sp1 transcription factor
SP3	Sp3 transcription factor
STAT1	signal transducer and activator of transcription 1
STAT3	signal transducer and activator of transcription 3
TAK1	mitogen-activated protein kinase kinase kinase 7 (MAP3K7)
TBK1	TANK binding kinase 1
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptors
TNFAIP3	TNF alpha induced protein 3
TNFα	tumor necrosis factor α
TOLLIP	toll interacting protein
TRAF1	TNF receptor associated factor 1
TRAF6	TNF receptor associated factor 6
TRIF	TIR domain-containing adaptor inducing interferon-β

1 Introduction

From birth, our skin is constantly exposed to various microbes, which are capable of transiently or permanently colonizing the cutaneous surfaces and forming the skin microbiota or microbiome. The microbiota has a dual role in our skin: in one hand, plays a crucial role in the maintenance of epidermal homeostasis, on the other hand, if the fine balance between the microbiota and skin cells are disturbed, dysbiosis may occur, and various members of this community have an impact in the pathogenesis of different skin diseases.

1.1 Skin anatomy and function

The skin is one of our largest organs, and its main function is to separate our body from the external environment. Because of its special anatomic structure and cell composition, it provides a complex mechanical, chemical and immunological barrier. Apart from that, the skin also has a role in temperature regulation, sensation, and protection against excessive water loss, UV light and pathogenic microbes (1).

The skin is composed of three layers (from top to bottom), the epidermis, dermis and the hypodermis (Figure 1.). The major cellular components of the epidermis are keratinocytes, forming a multilayered structure. Apart from keratinocytes, melanin-producing melanocytes, mechanosensitive Merkel cells, and tissue-resident macrophages, like Langerhans cells are also present in the epidermis.

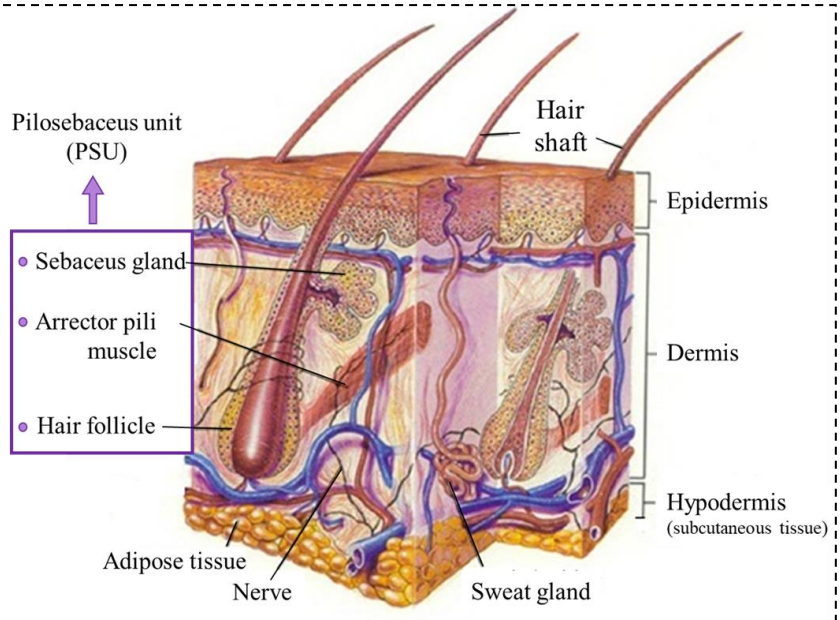

Figure 1. The anatomical structure of the skin

The dermis is connected to the epidermis by a basement membrane and consists of connective tissue. It contains hair follicles, different glands (sebaceous, sweat and apocrine glands), nerve endings, lymphatic and blood vessels.

The deepest layer of the skin is the hypodermis or subcutaneous tissue. It lies below the dermis and composed of loose connective tissue. It contains adipocytes, fibroblasts, and macrophages. Its main function is to serve as energy storage and plays a role in thermoregulation (2).

1.2 Localization and composition of the cutaneous microbiota

The cutaneous microbiota populates the epidermis, and the specialized anatomical and functional organelles called pilosebaceous units (PSUs) or follicles of the human skin (3,4). To date, approximately 1000 bacterial species belonging to 19 phyla, as well as fungal (dermatophytes) and viral species have been identified as members of this community (15). *Actinobacteria* (*Cutibacterium* and *Corynebacterium species*), *Proteobacteria*, *Firmicutes* (*Staphylococcus species*) and *Bacteroidetes* are the most common representatives of the four dominant bacterium phyla (3,5). At a species level, *Staphylococcus epidermidis* (*S. epidermidis*) and *Cutibacterium acnes* (*C. acnes*, formerly known as *Propionibacterium acnes*, *P. acnes*) are the most abundant and common members (6,7).

The human body has regional alterations in terms of pH, temperature, moisture, and the fine anatomic structure of the skin (thickness, sebaceous gland density), and these variations also influence the local composition of the cutaneous microbial communities. For example, in sebaceous gland rich skin regions, such as the back, face, and chest, the predominant bacterial species are the Gram-positive *Cutibacterium spp*, whereas moist areas favor the growth of *Staphylococcus* and *Corynebacterium* spp. Dry areas have the most complex bacterial composition, with representatives from all four bacterial phyla (3,5,8–10). Apart from these variations, many other individual lifestyle and environmental factors shape the microbial diversity of our skin, among others, the mode of birth, age, gender, geographical and socioeconomic properties (11).

1.3 The role of cutaneous microbiota in healthy skin and the pathogenesis of different skin diseases

The cutaneous microbiota contributes to the formation and maintenance of skin homeostasis in different ways. Resident microbes inhibit the growth of pathogenic microbes by competing for nutrients and inhabitant space and by the production of antimicrobial compounds(12). For example, *C. acnes*, which is one of the most common members of the skin’s resident microbial community exhibits a complex effect. It produces different

compounds with bacteriostatic and/or antimicrobial properties, including acnecin and short-chain fatty acids (SCFAs). The latter ones can control the growth of pathogenic microbes, such as the methicillin-resistant *Staphylococcus aureus* (*S. aureus*) bacterium and also inhibits the commensal *S. epidermidis* (13,14). On the other hand, the presence of the microbiota also leads to immune and inflammation activation of the host. As a consequence, the affected cell types release various cytokines, chemokines and small host molecules exhibiting antimicrobial properties, called antimicrobial peptides (AMPs), including human β defensins (hBDs) (15). The continuous presence of the skin microbiota keeps our immune system at a basal activated state, which helps to induce a rapid and stronger immune activation upon pathogenic attacks (12,16).

Various members of the microbiota are also important in the pathogenesis of different skin diseases. When they develop, microbial dysbiosis leads to exaggerated innate immune and inflammatory events. Examples of such diseases, where different members of the cutaneous microbiota play an important etiopathogenic role include *Malassezia* spp. in seborrheic dermatitis, *S aureus* in atopic dermatitis and *C. acnes* in acne vulgaris (11,17).

1.4 Skin microbiota-induced innate immune activation in keratinocytes

Keratinocytes are the major cellular components of the epidermis, and this particular cell type forms a continuous and direct contact with the different members of the cutaneous microbiota (5). Keratinocytes are immunocompetent cells, they can sense danger signals, known as pattern- or damage-associated molecular patterns (PAMPs and DAMPs), through their pattern recognition receptors (PRRs) (18,19). To date, the most well-known and studied PRRs are the Toll-like receptors (TLRs). PAMPs and DAMPs released by human and microbial cells upon insults or injury induce TLR activation, and subsequent activation of innate immune and inflammatory responses in keratinocytes (19,20).

1.4.1 *C. acnes* recognition by human epidermal keratinocytes and their role in acne pathogenesis

Epidermal keratinocytes recognize the presence of *C. acnes* bacterium in their environment through the activation of TLR2 and TLR4, induction of the canonical TLR signaling pathway, and subsequent innate immune and inflammatory events (21,22). Important mediators of this cascade are the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinases (p38) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinases (MAPKs), which regulate the expression of key genes, playing a role in the initialization and execution of downstream responses (23–25). These genes include different cytokines, such as tumor necrosis factor α (TNFα), interleukin (IL)-1α, IL-1β, and IL-6, chemokines, including the IL-8 and C-C motif chemokine ligand 5 (CCL5), antibacterial peptides, such as human beta-defensin 2 (hBD2), and other inflammatory mediators (26,27). The innate immune activation of keratinocytes and the inflammatory milieu they generate in their environment favors the activation of other cell types, including sebocytes, dendritic cells, and macrophages. Adaptive immune events are also induced, leading to the activation of the Th1/Th17 pathway (28,29). Overall, these events contribute to the induction of the characteristic inflammatory symptoms during acne vulgaris pathogenesis in adolescents (Figure 2).

Apart from the microbial component, several intrinsic and extrinsic factors also contribute to the pathogenesis of the diseases. Such intrinsic factor is the hormonal changes during puberty (mainly androgen excess), and as a consequence, the appearance of abnormal keratinocyte and sebocyte functions (enhanced epidermal keratinocyte proliferation, differentiation and increased sebum production of sebocytes) (30). Genetic predisposing factors, *e.g.* single nucleotide polymorphisms (SNPs) in different genes may also modify an individual risk to develop more severe forms of the disease (31).

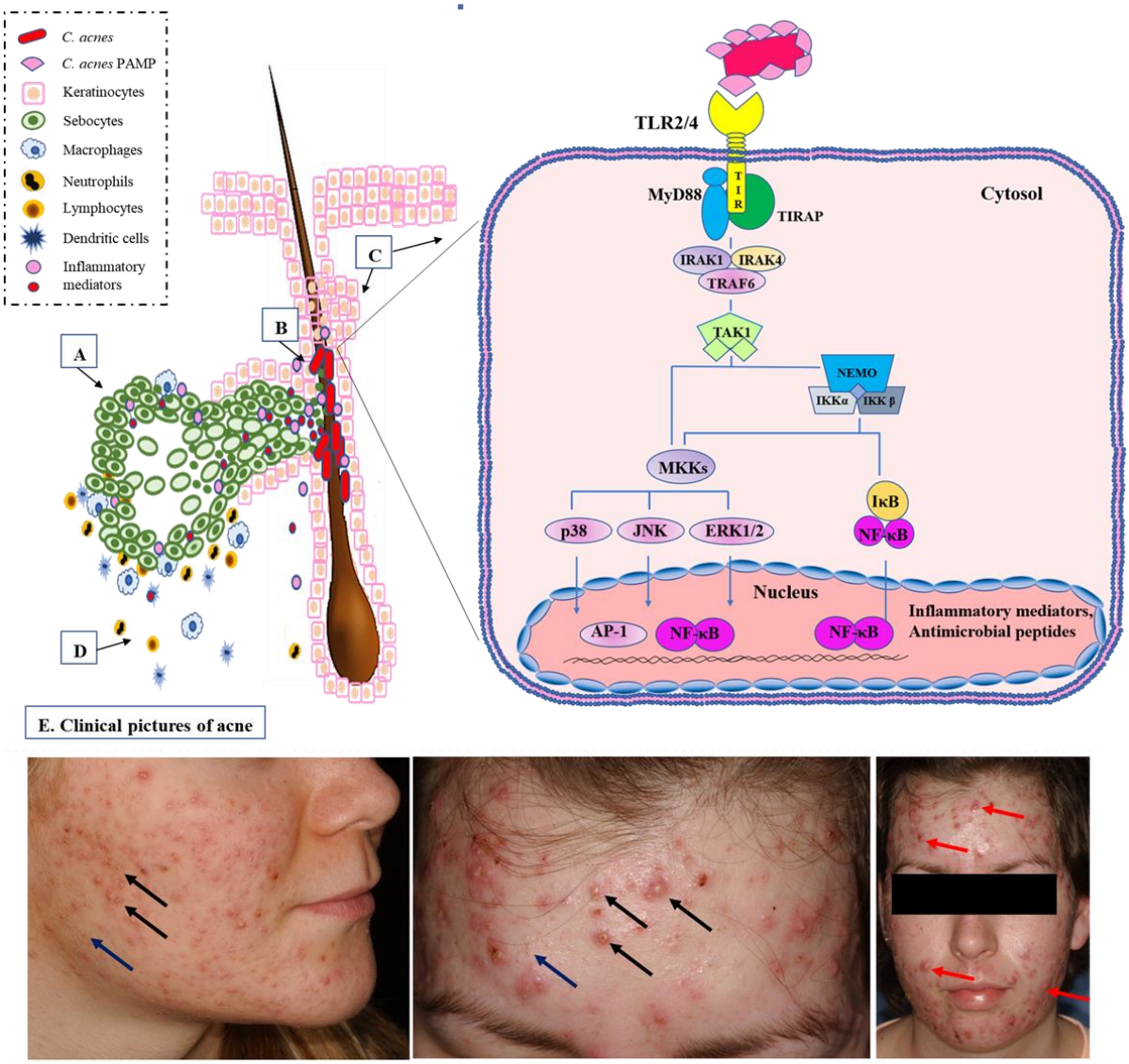

Figure 2. *C. acnes*-induced TLR signaling pathways in epidermal keratinocytes. **(A)** Increased sebum production of sebocytes, as a consequence of hormonal changes during puberty, favors **(B)** *C. acnes* proliferation. **(C)** Enhanced keratinocyte proliferation and differentiation can lead to keratinocyte hyperkeratosis. *C. acnes* recognition by TLR2 and TLR4 induces the canonical NFκB-dependent signaling pathways, increases the production of several cytokines and chemokines, antimicrobial peptides and other inflammatory mediators. **(D)** The generated inflammatory milieu favors the recruitment and the activation of other cell types, such as professional immune cells. Overall, these events contribute to the induction of the characteristic inflammatory symptoms **(E)** (comedos-blue arrows, papules- black arrows, pustules, and cysts- red arrows) during acne vulgaris pathogenesis. (Photos were selected from the collection of the Department of Dermatology and Allergology, University of Szeged.)

1.5 Factors contributing to the negative regulation of TLR signaling pathways

Inflammation and acne lesions are generally present transiently throughout life. In adolescents, the inflamed follicles heal by themselves and the affected individuals often do not exhibit any residual signs. After the resolution of the disease, *C. acnes* bacterium still dominates the microflora, especially in the sebum-rich skin regions, but the bacterium does not usually provoke immune activation and inflammation in keratinocytes and/or other immune cells (8,16,32). This age-dependent response to *C. acnes* indicates the existence of different mechanisms controlling the bacterium induced immune events, but currently, the exact nature of these regulatory mechanisms is still not known (33).

Control of innate immune activation is the key to avoid excessive inflammation and tissue damage. Several negative regulators of the TLR signaling pathways have been identified in the past decade, which act at different levels of the cascade (34,35). TLR pathways are suppressed, among others, by receptor interference (*e.g.*, soluble TLRs, single Ig and TIR domain containing - SIGIRR and interleukin 1 receptor like 1 - IL1RL1), inhibition and destabilization of adaptor complex formation (*e.g.*, SIGIRR, TNFAIP3 interacting protein 1-TNIP1, a short form of MYD88 innate immune signal transduction adaptor - MYD88, toll interacting protein - TOLLIP), by dampening crucial enzymatic modification steps (*e.g.*, TNF alpha induced protein 3 - TNFAIP3, interleukin 1 receptor-associated kinase 3 – Irak3, suppressor of cytokine signaling 1 - SOCS1) or by transcriptional regulation (miRNAs, including MIR146A, MIR155, MIR199) (36,37).

1.5.1 SIGIRR, TOLLIP, TNFAIP3 and TNIP1

Currently, it is not clear whether and how, and exactly which negative regulators control the human microbiome-induced downstream events. Thus, in our studies we selected SIGIRR, TOLLIP, TNIP1, and TNFAIP3, acting at different levels of the TLR signaling cascade (Figure 3).

SIGIRR is an orphan receptor, composed of a single extracellular Ig domain, a transmembrane domain, a cytoplasmic two amino acid modified TIR domain and a long tail. It controls the activation of different TLR, and the Interleukin 1 Receptor Type 1 (IL-1R1) signaling pathways by competing with the formation of MyD88 dimers, through their TIR domains. As a result, the activation of downstream signaling molecules (IRAK and TRAF6) is reduced, leading to NF-κB and JNK inhibition (38,39). Through this, SIGIRR plays

important roles in the regulation of inflammation, cell cycle, differentiation and cell homeostasis (40). Its role in the microbiota-induced processes is also suggested, SIGIRR deficient mice were found to be susceptible to intestinal *Salmonella typhimurium* colonization, parallel with the loss of commensal microbes in the intestine (41).

TOLLIP is another negative regulator of TLR and IL-R1 signaling pathways. By direct interaction with TLR2 and TLR4, it inhibits downstream signaling and prevents the subsequent inflammatory responses (42,43). It blocks the auto-phosphorylation of IRAK1 and IRAK-2 by direct binding, thus dampens the activation of NF-κB and the production of inflammatory cytokines (42). Consequentially, TOLLIP facilitates the clearance of cellular stress molecules and helps to restore cellular homeostasis (44).

TNFAIP3, also known as A20, is an ubiquitin editing enzyme. Through its zinc finger domains, a ubiquitin ligase, and deubiquitinase activity, TNFAIP3 controls TLR and TNFR pathways at different levels (45). TNFAIP3 acts directly on RIP1 and NEMO, which causes the termination of NF-κB activation, and subsequently dampens the downstream signaling cascades (46). TNFAIP3 functions in human keratinocytes, and appears to be a general regulator of immune and inflammatory cascades in response to different ligands, for example, poly-I:C and imiquimod (TLR3 and TLR7 ligands, respectively) (47,48). A recent study also suggested that TNFAIP3 may help *S. epidermidis* to persist as a commensal on the human skin, through the control of bacterium-induced NF-κB activation, IL-1β and hBD2 production in keratinocytes (49).

TNIP1, also known as ABIN1, was identified as a TNFAIP3 interacting partner. It can affect different signaling pathways by interacting directly with various proteins, including NEMO, TRAF1, p105, FADD, and RIP1. As a consequence, the TLR-MYD88 signaling cascade and the NF-κB transcription factor is inhibited, and apoptotic and autoimmune events are negatively affected (50,51). TNIP1 is also expressed in keratinocytes, where this protein controls cell proliferation partly due to the regulation of ERK1/2 signaling cascades (52), and its attenuation sensitizes HaCaT keratinocytes to synthetic TLR ligand treatments (53).

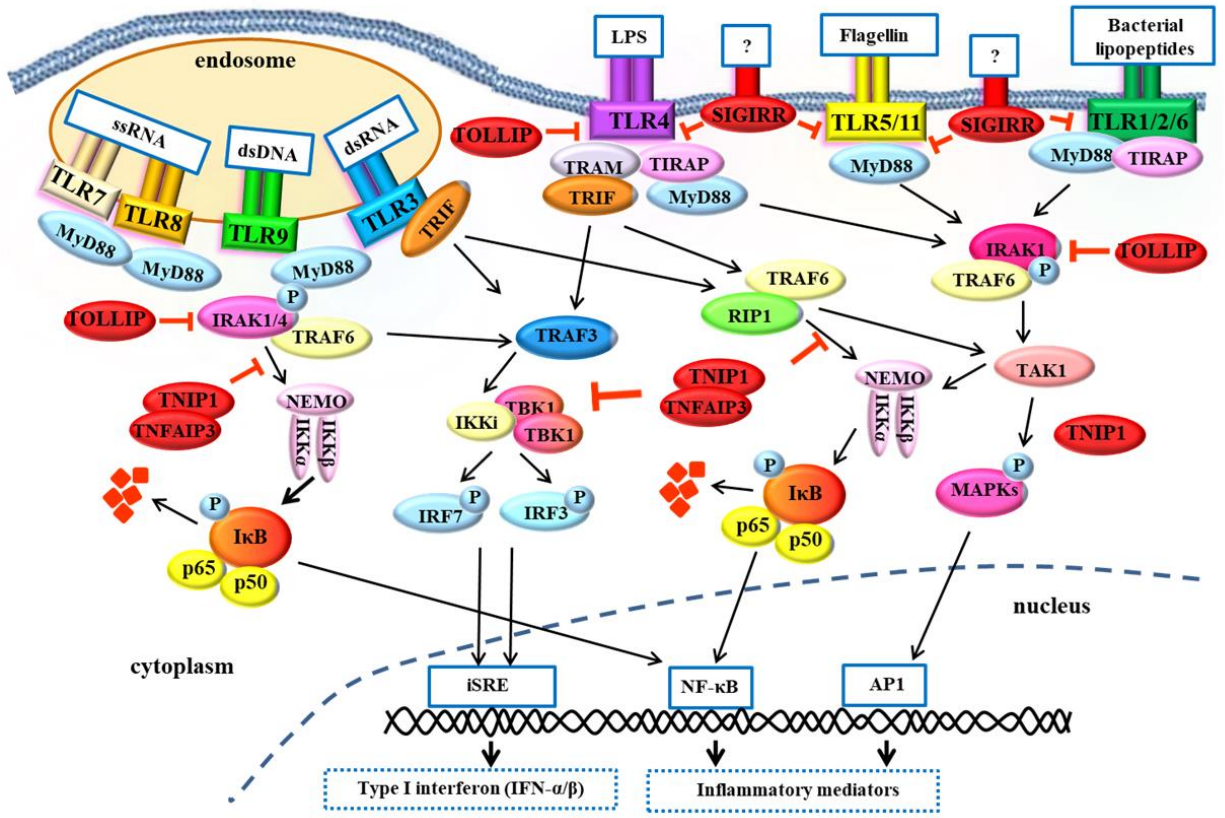

Figure 3. Negative regulation of TLR signaling pathways by SIGIRR, TOLLIP, TNFAIP3 and TNIP1. Adapted from *Zhu and Mohan, Mediators Inflamm, 2010*. (54). (SIGIRR, TOLLIP, TNFAIP3 and TNIP1 are highlighted with red.)

2 Aims

Our main objective was to identify factors and signaling pathways which play a regulatory role in the microbiota, especially *C. acnes*-induced innate immune activation in human epidermal keratinocytes.

For that, we aimed to analyze well-known negative regulators of TLR signaling pathways, including SIGIRR, TOLLIP, TNFAIP3, and TNIP1:

- whether their expression changes in response to *C. acnes* in keratinocytes,
- if selected *C. acnes* strains, belonging to various phylogenetic groups within the species (889: 1A, 6609: 1B, ATCC 11828: II), differentially alter the expression of these factors,
- whether and how their expression changes modify the *C. acnes*-induced innate immune and inflammatory events in a human immortalized keratinocyte cell line, HPV-KER,
- and exactly how the expression of these factors is regulated in keratinocytes.

3 Materials and Method

3.1 Cell cultures and models

The human immortalized keratinocyte cell line, HPV-KER, was used for our experiments (55). Cells were cultured in keratinocyte serum-free medium (KSFM, Life Technologies, Carlsbad, USA) containing 1% antibiotic/antimycotic (AB/AM) (Sigma Aldrich, St. Louis, MO, USA) solution and supplemented with epidermal growth factor and brain pituitary extract under standard laboratory conditions (37°C in a humidified atmosphere containing 5% v/v CO_2).

Normal human epidermal keratinocytes (NHEK) and *ex vivo* skin biopsies were taken from skin specimens obtained from the Plastic Surgery Unit of our Department. Written informed consent was obtained from investigated individuals. The study was approved by the Human Investigation Review Board of the University of Szeged (PSO-EDAFN-002, 34/2015, 3517, 23 February 2015, Szeged, Hungary) and complied with the ethical standards of research and was in accordance with the Helsinki Declaration.

NHEK cells were isolated from skin samples. Briefly, the adipose tissue was trimmed away and the epidermis and dermis were separated by overnight dispase digestion (Roche Diagnostics, Manheim, Germany) at 4 °C. Keratinocytes were isolated with 0.25% trypsin (Sigma Aldrich, St. Louis, MO, USA), cultured in a T75 flask and used at the third passage for subsequent experiments.

For *ex vivo* organotypic skin (OS) cultures, 1 cm x 1 cm biopsies were washed first with normal saline solution (NSS) containing 2% AB/AM, followed by a wash with AB/AM-free NSS. Subsequently, the biopsies were placed onto the upper chamber of Transwell® Inserts (Corning, New York, USA) and kept at the air–liquid interphase. The dermal part of the biopsies was in contact with DMEM F12 liquid culture medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (EuroClone, Milan, Italy) lacking AB/AM.

3.2 Treatments

For bacterial treatment, HPV-KER and NHEK cells were plated in AB/AM-free KSFM culture medium and co-cultured with live *C. acnes* strains belonging to different phylogenetic groups within the species (889, I/A, ATCC 11828, II, 6609, I/B) for various time using different multiplicity of infection (MOI). *Ex vivo* skin models were treated with the *C. acnes*

889 strain at a density of 3 x 10^7 colony-forming units (cfu) per cm^2 for 24 hours. *C. acnes* were cultured on pre-reduced Columbia agar base supplemented with 5% (v/v) bovine blood, vitamin K1 and haemin (Oxoid, UK). Bacteria were grown at 37 °C under anaerobic conditions. Single bacteria colonies were inoculated in brain heart infusion (BHI, pH 7.4; Oxoid, UK) and incubated at 37 °C for 48 hours. After centrifugation (2600 g, 10 minutes, 4 °C) and supernatant discard, *C. acnes* were resuspended in PBS and the number of bacterial cells was measured with a density at 600 nm. The number of bacterial cells was adjusted to 1 × 10^9 cfu/ml with PBS and aliquoted and stored at −80°C until further use. Every experiment was performed with fresh aliquots.

To analyze the effect of the active form of retinoic acid, ATRA was dissolved in DMSO and a 10^{-6} M concentration was applied to HPV-KER cells for 48 h before *C. acnes* challenge. ATRA was applied to OSs at a 1.5 × 10^{-6} M concentration for 24 h. As a control, cells were subjected to DMSO treatment without the active ingredient.

The selective inhibitors of JNK (sp 600125, 10 μM), NF-κB (Bay 11-7085, 10 μM), p38 (sb 203580, 10 μM), ERK1/2 (PD 098059, 20 μM), STAT1 (Fludarabine, 25 μM) and STAT3 (Stattic, 5 μM) or, as a control, DMSO were applied to the cells for 1 hour (all reagents from Sigma Aldrich, St. Louis, MO, USA).

3.3 Transfection, plasmids and siRNA-mediated gene silencing

Transient transfection experiments were performed using the X-tremeGENE 9 DNA Transfection Reagent (Roche, Indiana, USA). For the overexpression studies, HPV-KER cells were plated in 12-well plates (100,000 cells/well), transfected for 24 hours with 0.5 μg empty pcDNA3.1 vector or pcDNA3.1-TNIP1 vector into which TNIP1 cDNA sequences (OriGene Technologies, Inc., MD, USA) had been inserted. Transfection-grade plasmid was prepared using the E.Z.N.A Endo-free plasmid DNA Maxi Kit (Omega Bio-tek, Inc., GA, USA).

For transient siRNA-mediated gene silencing, siRNA was delivered by the Santa Cruz siRNA Transfection Reagent (Santa Cruz Biotechnology, Texas, USA) according to the manufacturer's instructions. 25 nM ON-TARGETplus SMARTpool TNFAIP3-siRNA, 10 nM TNIP1-siRNA, SIGIRR-siRNA, TOLLIP si-RNA or ON-TARGETplus Non-targeting Pool (Dharmacon, Lafayette, USA) constructs were used at the same concentration for 48 hours.

NF-κB luciferase reporter assay was performed using the PathDetect pNF-κB-Luc Cis-Reporter Plasmid (Stratagene, California, USA), pGL4.75[hRluc/CMV] vector, which was

transfected with X-tremeGENE 9 DNA Transfection Reagent (Roche, Indiana, USA). For analysis, the Firefly & Renilla Dual Luciferase Assay Kit (Biotium Inc, California, USA) was used according to the manufacturer's instructions.

3.4 ELISA

C. acnes-treated and control HPV-KER cell culture supernatants were collected and levels of secreted IL-8, IL-6 and CCL5 were measured by enzyme-linked immunosorbent assay (ELISA, PeproTech EC Ltd., London, UK) according to the manufactures' instructions.

3.5 RNA isolation, cDNA synthesis and real-time RT-PCR

Total RNA from HPV-KER, NHEK cells or the epidermal part of OS cultures were isolated using TRI-Reagent (Molecular Research Center; Cincinnati, USA). cDNA synthesis was performed using 1 μg RNA with the iScript TM cDNA Synthesis kit (Bio-Rad, Hercules, USA). Changes in mRNA expression were detected by real-time RT-PCR using the Universal Probe Library (Roche, Indiana, USA) or the TaqMan Gene Expression Assay (Thermo Scientific, Rockford, USA). Table 1. lists the PCR protocols and primer sequences used. All data were normalized to the 18S rRNA using the $\Delta\Delta C_t$ method and compared to the time-matched untreated control samples.

Gene	Primer sequences	Probe	PCR protocol			
			Denaturation	Annealing	Extension	Cycle
18S rRNA	FWD: CGCTCCACCAACTAAGAACG REV: CTCAACACGGGAAACCTCAC	77	95°C	58°C	37°C	40x
TNFAIP3	FWD: TGCACACTGTGTTTCATCGAG REV: ACGCTGTGGGACTGACTTTC	74				
TNIP1	FWD: TGGTCACGCAGAATGAGTTG REV: CTCCCTCTGGAAGTCCTCCT	62				
SIGIRR	FWD: CTCAGAGCCATGCCAGGT REV: CCTCAGCACCTGGTCTTCA	55				
TOLLIP	FWD:TCCCCGCTGGAATAAGGT REV: CGTCCATGGAGAAGGCTCT	86				
IL-6	FWD: CAGGAGCCCAGCTATGAACT REV: GAAGGCAGCAGGCAACAC	45				
IL-1A	FWD: GGTTGAGTTTAAGCCAATCCA REV: TGCTGACCTAGGCTTGATGA	6				
CCL5	FWD: TGCCCACATCAAGGAGTATTT REV: CTTTCGGGTGACAAAGACG	59				
TLR2	FWD: CTCTCGGTGTCGGAATGTC REV: AGGATCAGCAGGAACAGAGC	56				
TLR3	FWD: AGAGTTGTCATCGAATCAAATTAAAG REV: AATCTTCCAATTGCGTGAAAA	80				
TLR4	FWD: GCCTGATTTCATTTATATGAGGTTCTA REV: GACAACTCCCTATTTCCTCATTTCT	69				
TNFA	Taq Man Genexpression assay Hs00174128_m1			60°C		
IL-8	Taq Man Genexpression assay Hs001743103					

Table 1. Real-time RT-PCR primers and the applied protocol.

3.6 Protein isolation and western blot analysis

For the preparation of whole cell lysates, samples were collected and lysed in lysis buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 5% Triton X-100, 10% glycerol, 0.1% NP-40, 1% Protease Inhibitor Cocktail, phenylmethylsulfonyl fluoride and 0.5% SDS (all from Sigma Aldrich, St. Louis, MO, USA). Protein concentrations were measured with the BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Samples (30 μg) were separated on a 7.5% SDS-PAGE gel and transferred to nitrocellulose membrane (Bio-Rad, Hercules, USA), blocked in Tris-buffered saline containing 5% non-fat dried milk. Membranes were incubated overnight at 4°C with primary anti-TNFAIP3 antibody diluted 1:1000 (Abcam, Cambridge, United Kingdom), anti-SIGIRR antibody diluted 1:1000, anti-TOLLIP antibody diluted 1:1000 anti-TNIP1 antibody diluted 1:500 and anti-actin (Sigma Aldrich, St. Louis, MO, USA) diluted 1:1000. Subsequently, membranes were incubated for 1 hour at room temperature with horseradish peroxidase- or alkali-phosphatase–conjugated anti-rabbit or anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Texas, USA) diluted 1:2000. Proteins were visualized with 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) (Sigma Aldrich, St. Louis, MO, USA) or with luminol (Bio-Rad, Hercules, USA) using an Omega Lum™ G Imaging System (Gel Company, CA, USA) and quantified using Image Pro Plus Software.

3.7 Fluorescence microscopic analysis

HPV-KER cells were grown on glass sides, fixed with 2% paraformaldehyde (PFA) for 5 minutes, permeabilized with 0.1% Triton X, 2% PFA-containing phosphate-buffered saline (PBS), and blocked for 2 hours at room temperature (RT) with PBS containing 1% bovine serum albumin (BSA), 0.05% Triton X 100, and 10% goat serum. Cells were stained overnight at 4°C with anti-human TNIP1 antibody (Sigma Aldrich, St. Louis, MO, USA) or rabbit IgG for isotype control. As a secondary antibody, Alexa Fluor 488 conjugated anti-rabbit IgG, was used for 2 hours at RT. Filamentous actin was stained by Alexa Fluor 546® phalloidin (Life Technologies, Carlsbad, USA) diluted 1:100 in PBS containing 1% BSA for 20 minutes. Nuclei were stained for 10 minutes with 4',6-diamidino-2-phenylindole (DAPI) diluted 1:500. TNIP1 were visualized with Zeiss Axio Imager Z1fluorescence microscope.

Frozen sections of *ex vivo* skin models were pre-incubated with PBS for 5 minutes and fixed and permeabilized with Foxp3 staining buffer set (Thermo Scientific, Rockford, USA)

and blocked for 1 hour at RT with Tris-buffered saline (TBS) containing 1% BSA and 1% normal goat serum (NGS) (Sigma Aldrich, St. Louis, MO, USA). Cells were stained for 1 hour with anti-human TNIP1 antibody or rabbit IgG for isotype control. As a secondary antibody, Alexa Fluor 546 conjugated anti-rabbit IgG (Thermo Scientific, Rockford, USA) was used for 1 hour at RT. Nuclei were stained for 6 minutes with 4′,6-diamidino-2-phenylindole (DAPI) diluted 1:100. TNFAIP3 was visualized using a ZEISS LSM 880 Confocal Laser Scanning Microscope (Zeiss, Oberkochen, Germany), magnification: 63x.

For visualization of TNFAIP3, frozen sections of *ex vivo* OS models were pre-incubated with PBS for 5 minutes, fixed with 4% paraformaldehyde for 5 minutes and permeabilized with 0.25% TritonX-100 (Thermo Scientific, Rockford, USA) containing TBS for 10 minutes. For blocking, TBS containing 5% FBS and 5% NGS was used for 1 hour at RT. Cells were stained for 1 hour with anti-human TNFAIP3 antibody diluted 1:300 or rabbit IgG (Santa Cruz Biotechnology, Texas, USA) for isotype control. As a secondary antibody, Alexa Fluor 546 conjugated anti-rabbit IgG (Thermo Scientific, Rockford, USA) was used for 1 hour at RT. Nuclei were stained for 5 minutes with DAPI diluted 1:100. TNFAIP3 was visualized using a ZEISS LSM 880 Confocal Laser Scanning Microscope (Zeiss, Oberkochen, Germany) magnification: 63x.

3.8 Data analysis

TNFAIP3 gene expression was analyzed using publicly available microarray data from the GEO Profile Database (https://www.ncbi.nlm.nih.gov/geoprofiles/33444972, GDS2478 datasets, ID: 33444972), which compares the total RNA content of healthy skin (n=6) with lesional (n=6) and non-lesional (n=6) samples from acne patients (56).

3.9 Statistical analysis

Unless otherwise noted, all data are presented as mean ± standard error of the mean (SEM) of three independent experiments. For real time RT-PCR analyzes and ELISA, each treatment was performed at least in triplicate; for western blot and fluorescence microscopic analysis, each treatment was performed once in every independent experiment. Data were compared using paired, two-sample t-test with Holm–Bonferroni or false discovery rate (FDR) correction using RStudio. A probability value of less than 0.05 was considered significant.

4 Results

We aimed to systematically analyze SIGIRR, TOLLIP, TNFAIP3 and TNIP1 mRNA and protein expression changes in response to *C. acnes* bacterium and studied their possible role in the bacterium-induced innate immune activation of keratinocytes.

4.1 Studying the expression levels and the possible role of SIGIRR in the *C. acnes*-induced innate immune activation of keratinocytes

We analyzed SIGIRR expression changes in human *in vitro* cultured keratinocytes co-cultured with the *C. acnes* 889 strain (MOI=100). The applied *C. acnes* dose was determined in extensive preliminary studies. We chose conditions that induced relatively fast and reproducible cellular responses but did not induce cell death in the time course of our studies (55). We found that SIGIRR is expressed in HPV-KER cells, but its mRNA expression remained unchanged after *C. acnes* treatment (Figure 4).

It has been suggested that various *C. acnes* strains may have different effects on the cellular and molecular properties of human keratinocytes (27,55,57). We applied *C. acnes* strains, belonging to various phylogenetic groups within the species (*C. acnes* 889, 6609, ATCC 11828, Group IA, IB and II.) (MOI=100) and compared the effects. None of the applied strains induced SIGIRR mRNA expression changes at the used bacterial doses (Figure 4/A, B, C).

To analyze whether SIGIRR expression depended on the used *C. acnes* doses, we applied the bacterium in different MOIs (25, 100, 300) for 6 hours, but SIGIRR mRNA expression did not change during the time-course of the experiment (Figure 4/D).

We also studied SIGIRR protein expression changes upon treatment with the *C. acnes* 889 strain. Similarly to the mRNA results, its protein levels remained the same in the presence of the bacterium.

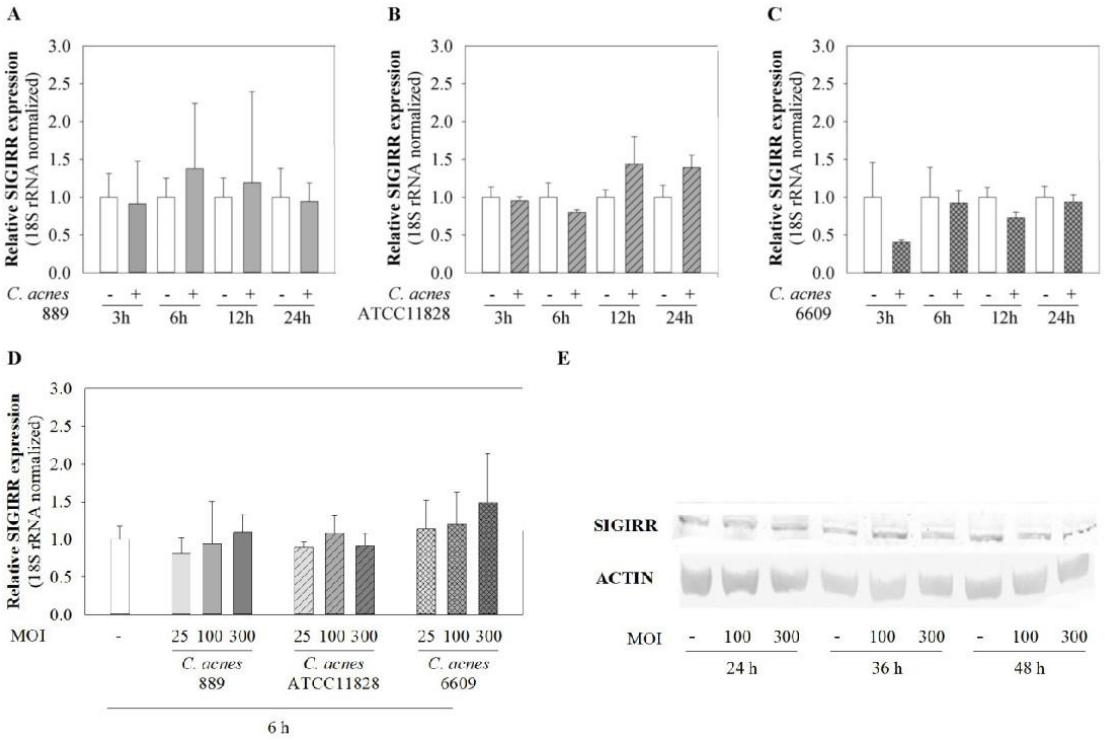

Figure 4. SIGIRR mRNA and protein expression levels do not change in HPV-KER cells, co-cultured with selected *C. acnes* strains. HPV-KER cells were treated with the *C. acnes* 889, ATCC11828, and 6609 strains (MOI=100, unless otherwise indicated). **(A-D)** SIGIRR mRNA expression changes were studied by real-time RT-PCR. Error bars are SD, representative experiment. **(E)** SIGIRR protein was detected with western blot analysis. Representative blot.

Next, we also tested whether changes in SIGIRR levels modify the *C. acnes*-induced innate immune and inflammatory events in HPV-KER cells. For that, transient siRNA-mediated silencing was performed, and *C. acnes*-induced TNFα mRNA expression changes were monitored by real-time RT-PCR. Silencing of SIGIRR resulted in a 60% decrease in the mRNA levels, but neither basal nor the bacterium-induced TNFα levels were affected (Figure 5/A, B).

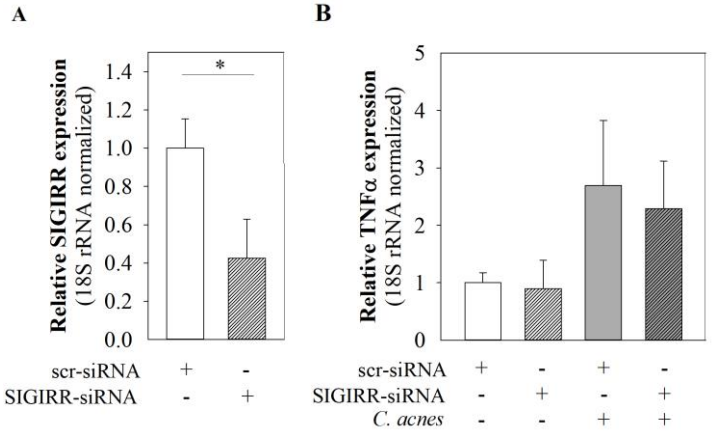

Figure 5. SIGIRR expression changes do not affect TNFα mRNA expression levels. siRNA-mediated silencing (48 hours) of SIGIRR was performed in HPV-KER cells, and they were co-cultured with the *C. acnes* 889 strain (MOI=100) for 6 hours. **(A, B)** SIGIRR and TNFα mRNA levels were analyzed by real-time RT-PCR. All data were normalized to 18S rRNA and compared to the time-matched untreated samples transfected with scrambled siRNA (scr-siRNA). Error bars are SD, representative experiments. Statistical analysis: paired, two-sample t-test, * $p<0.05$.

Our results suggest that even though SIGIRR is expressed in HPV-KER cells, it does not seem to play important roles in the negative regulation of the *C. acnes*-induced immune and inflammatory processes in keratinocytes.

4.2 Studying the expression levels and the possible roles of TOLLIP, in the *C. acnes*-induced innate immune activation of keratinocytes

Next, we analyzed TOLLIP expression levels in HPV-KER cells upon *C. acnes* treatment. We co-cultured the cells with the *C. acnes* 889, 6609 and ATCC 11828 strains using different bacterial doses and analyzed TOLLIP expression by real-time RT-PCR. We found that TOLLIP mRNA levels remained unchanged using this experimental setup (Figure 6/A, B, C, D). We observed similar results by analyzing TOLLIP protein levels upon *C. acnes* 889 treatment (Figure 6/E).

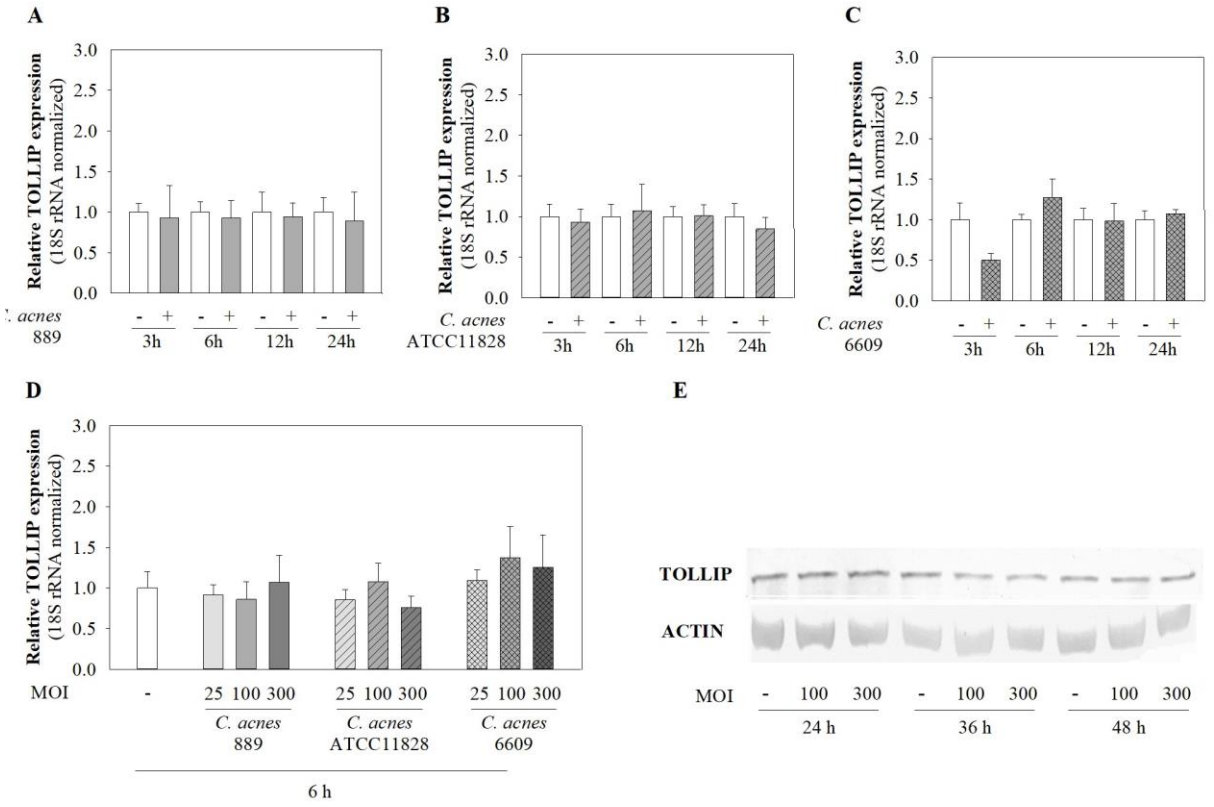

Figure 6. TOLLIP mRNA and protein expression levels do not change in HPV-KER cells, co-cultured with selected *C. acnes* strains. HPV-KER cells were treated with the *C. acnes* 889, ATCC11828, and 6609 strains (MOI=100, unless otherwise indicated). **(A-D)** TOLLIP mRNA expression changes were studied by real-time RT-PCR. Error bars are SD, representative experiment. **(E)** TOLLIP protein was detected with western blot analysis. Representative blot.

We also investigated whether changes in TOLLIP levels modify the bacterium-induced TNFα levels in HPV-KER cells. We found that transient, siRNA-mediated silencing of TOLLIP did not influence basal and *C. acnes*-induced TNFα mRNA expression levels (Figure 7/A, B).

Our results suggest that similar to our observation regarding SIGIRR, TOLLIP is expressed in HPV-KER cells, but it does not seem to play important roles in the negative regulation of the *C. acnes*-induced processes in keratinocytes.

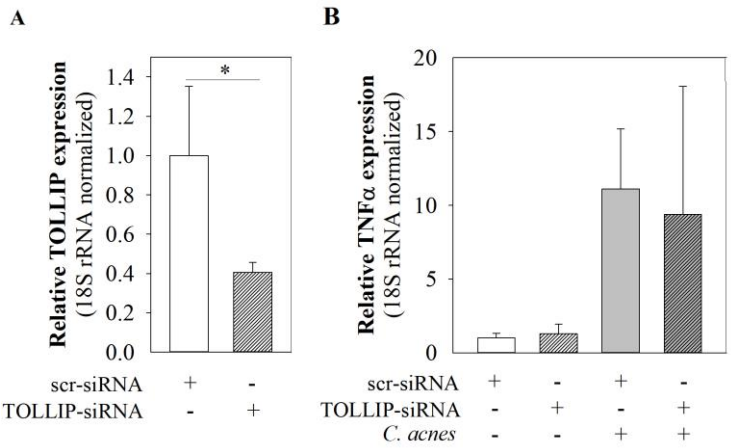

Figure 7. Changes in TOLLIP levels do not affect TNFα mRNA expression. siRNA-mediated silencing (48 hours) of TOLLIP was performed in HPV-KER cells, and they were co-cultured with the *C. acnes* 889 strain (MOI=100) for 6 hours. **(A, B)** TOLLIP and TNFα mRNA levels were analyzed by real-time RT-PCR. All data were normalized to 18S rRNA and compared to the time-matched untreated samples transfected with scrambled siRNA (scr-siRNA). Error bars are SD, representative experiments. Statistical analysis: paired, two-sample t-test, * $p<0.05$.

4.3 Studying the expression levels and the possible role of TNFAIP3 in the *C. acnes*-induced innate immune activation of keratinocytes

Next, we investigated the possible role of TNFAIP3 in the *C. acnes*-induced innate immune activation of keratinocytes.

Earlier studies have already shown that TNFAIP3 is expressed in the human epidermis (47). We confirmed this finding by immunofluorescence staining in healthy, full-thickness skin biopsy samples, where the TNFAIP3 protein was present throughout the entire epidermis, especially in the more differentiated keratinocyte layers. (Figure 8.)

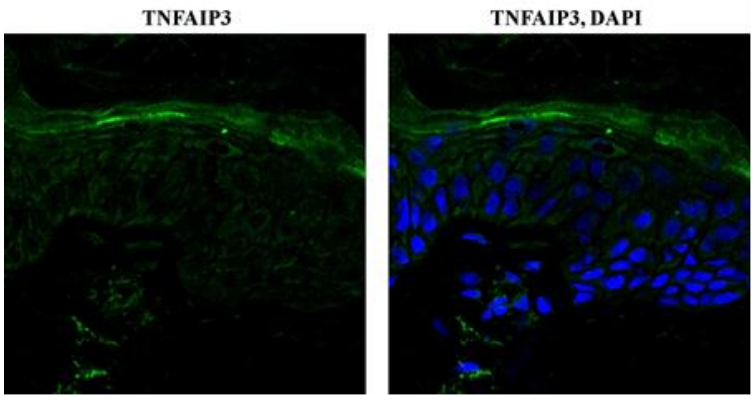

Figure 8. TNFAIP3 expression in the skin. TNFAIP3 protein expression was visualized using immunofluorescence staining for TNFAIP3 (green) and DAPI (blue) in a full-thickness skin biopsy.

4.3.1 *C. acnes* bacterium increases TNFAIP3 expression in keratinocytes

To analyze whether *C. acnes* affects TNFAIP3 expression levels in keratinocytes, we co-cultured HPV-KER cells with the *C. acnes* 889 bacterium strain (MOI=100) and analyzed the mRNA and protein expression changes with real-time RT-PCR and western blot analysis. We found that TNFAIP3 mRNA levels rapidly and transiently increased in the presence of the bacterium, reaching a maximum at 12 hours after bacterial treatment (Figure 9/A).

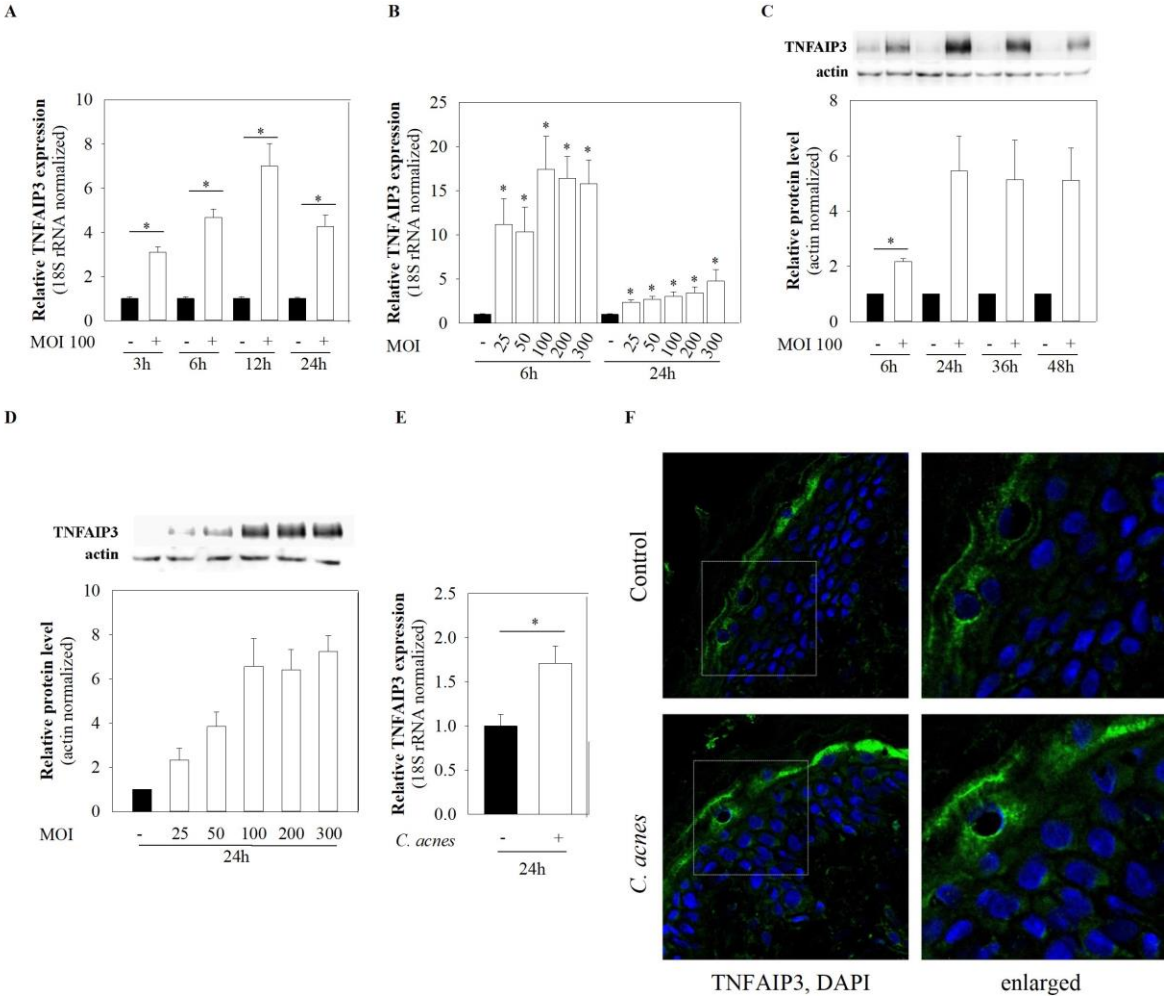

Figure 9. *C. acnes* upregulates TNFAIP3 mRNA and protein expression levels. **A-D)** HPV-KER cells were treated with the *C. acnes* 889 strain (MOI=100, unless otherwise indicated) and mRNA expression changes were analyzed by real-time RT-PCR, whereas protein expression was followed by western blot analysis. **(E, F)** OS samples were treated with the bacterium for 24 hours. mRNA expression of TNFAIP3 was analyzed only in the epidermis by real-time RT-PCR, and protein expression was visualized using immunofluorescence staining for TNFAIP3 (green) and DAPI (blue) in the full-thickness skin biopsies. mRNA data were normalized to 18S rRNA, whereas protein data to actin and compared to the time-matched untreated control samples. Error bars are SE. Statistical analysis: paired, two-sample t-test with Holm–Bonferroni correction: * $p<0.05$.

To analyze a possible strain-specific regulation of TNFAIP3, we co-cultured the HPV-KER cells with *C. acnes* 6609 and ATCC 11828 strains (MOI=100) and compared their effects. We did not observe any strain-specific differences: TNFAIP3 mRNA expression changes were similar, independent of the strain used (Figure 10).

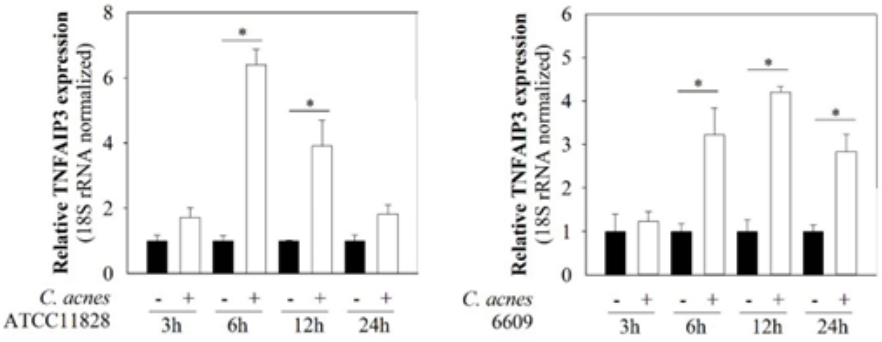

Figure 10. TNFAIP3 expression changes does not differ in HPV-KER cells, co-cultured with different *C. acnes* strains. HPV-KER cells were treated with *C. acnes* ATCC11828 or 6609 strains (MOI=100) and TNFAIP3 mRNA expression was analyzed by real-time RT-PCR (representative experiment). Data were normalized to 18S rRNA and compared to the time-matched untreated control values. Error bars are SD. Statistical analysis: paired, two-sample t-test, * $p<0.05$.

TNFAIP3 protein expression levels also rapidly increased in response to the bacterium (MOI=100) and remained high during the time-course of our study (Figure 9/C).

We also analyzed whether changes in *C. acnes*-induced TNFAIP3 expression were dose-dependent, by co-culturing HPV-KER cells with different MOIs. We found that the extent of TNFAIP3 mRNA and protein expression changes depended on the bacterial dose (Figure 9/B, D).

To confirm that the observed changes were not specific properties of the immortalized keratinocyte cell line used, we performed the experiments with established OS cultures. *Ex vivo* skin biopsies were treated with live *C. acnes* bacterium and changes in the mRNA expression were analyzed using real-time RT-PCR. TNFAIP3 mRNA expression levels increased in the epidermis part of the OS cultures upon *C. acnes* treatment, similar to what we observed in monolayer cultures. Immunofluorescence microscopy analysis also revealed elevated TNFAIP3 protein levels (Figure 9/E, F).

4.3.2 Changes in *C. acnes*-induced TNFAIP3 expression are dependent on JNK and NF-κB signaling pathways

We investigated the signaling mechanisms playing important roles in TNFAIP3 regulation. The DREAM transcription complex is known to repress its transcription when bound to the DRE3 and DRE4 elements in the TNFAIP3 promoter region in human lung microvessel endothelial cells (HLMVECs). The TNFAIP3 promoter also harbors functional NF-κB binding sites (58,59). To identify the possible signaling pathways that are involved in *C. acnes*-induced upregulation of TNFAIP3 in keratinocytes, we investigated the contribution of selected pathways playing important roles in the bacterium-induced signaling events (23–25). We inhibited the NF-κB and MAPK (JNK, p38 and ERK1/2) signaling pathways using specific inhibitors and analyzed changes in TNFAIP3 mRNA and protein expression in HPV-KER cells after co-culturing the cells for 12 and 24 hours with the *C. acnes* 889 strain.

We found that inhibition of the JNK signaling pathway decreased basal TNFAIP3 mRNA expression, whereas *C. acnes*-induced mRNA (Figure 11/A), and also protein (Figure 11/C) levels were affected by JNK and NF-κB inhibition.

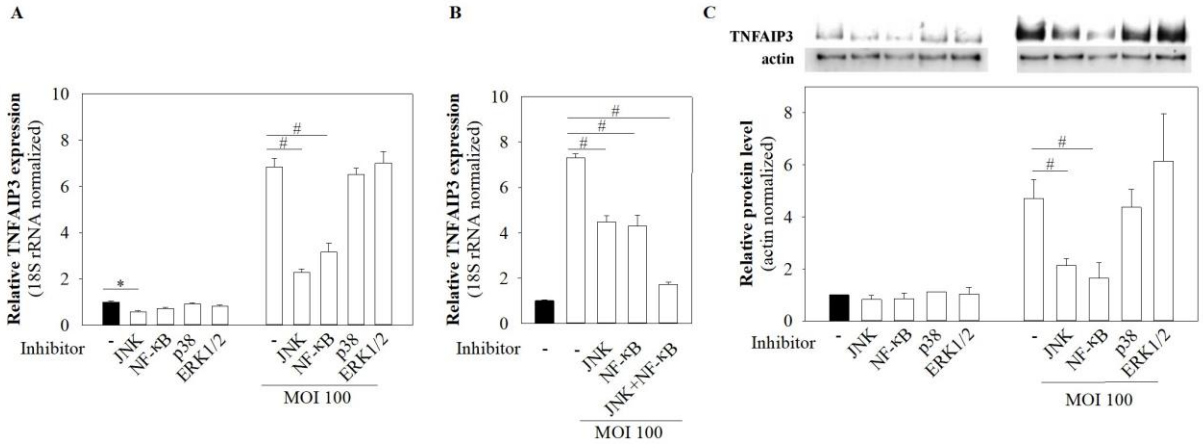

Figure 11. JNK and NF-κB regulates *C. acnes*-induced upregulation of TNFAIP3 expression in keratinocytes. HPV-KER cells were pretreated with selective inhibitors for JNK, NF-κB, p38, and ERK1/2 or, as a control, DMSO for 1 hour. **(A, B)** *C. acnes* challenge was performed for 12 hours and mRNA expression changes were analyzed by real-time RT-PCR. Data were normalized to 18S rRNA. **(E)** After a 24 hour bacterial treatment, protein expression was monitored by western blot analysis. Data were normalized to actin. All data were compared to time-matched DMSO-treated samples. Statistical analysis: paired, two-sample t-test with Holm–Bonferroni correction, where basal TNFAIP3 expression * <0.05 or *C. acnes*-induced expression # $p<0.05$ were compared.

Dual inhibition of these two signaling pathways almost completely prevented the bacterium-induced changes in mRNA expression (Figure 11/B), suggesting that these two pathways play major roles in the transcriptional regulation of the TNFAIP3 locus.

4.3.3 TNFAIP3 affects the basal and *C. acnes*-induced levels of inflammatory mediators in keratinocytes

To analyze the role of TNFAIP3 in the regulation of key inflammatory mediators, siRNA-mediated silencing was performed. First, we analyzed the effect of TNFAIP3 on NF-κB activity using a luciferase reporter assay. Silencing resulted in an average knockdown of 40% of TNFAIP3 protein levels (Figure 12/A). In these samples, the basal NF-κB promoter activity significantly increased, compared to the scrambled-siRNA (scr-siRNA) transfected controls (Figure 12/B).

We also monitored the consequences of these events and found that TNFAIP3 silencing significantly increased the basal mRNA expression of TNFα, IL-1α, IL-6, IL-8 and CCL5 and the secreted levels of IL-6 and IL-8, compared to the scr-siRNA control samples. These data suggest that TNFAIP3 may have important roles in the establishment and maintenance of keratinocyte cellular homeostasis by controlling the constitutive expression of different key inflammatory mediators.

We also investigated the effect of TNFAIP3 knockdown on the *C. acnes*-induced levels of IL-6, IL-8 and CCL5, and found that silencing also significantly increased the levels of these mRNAs and the secreted proteins (Figure 12/C, D).

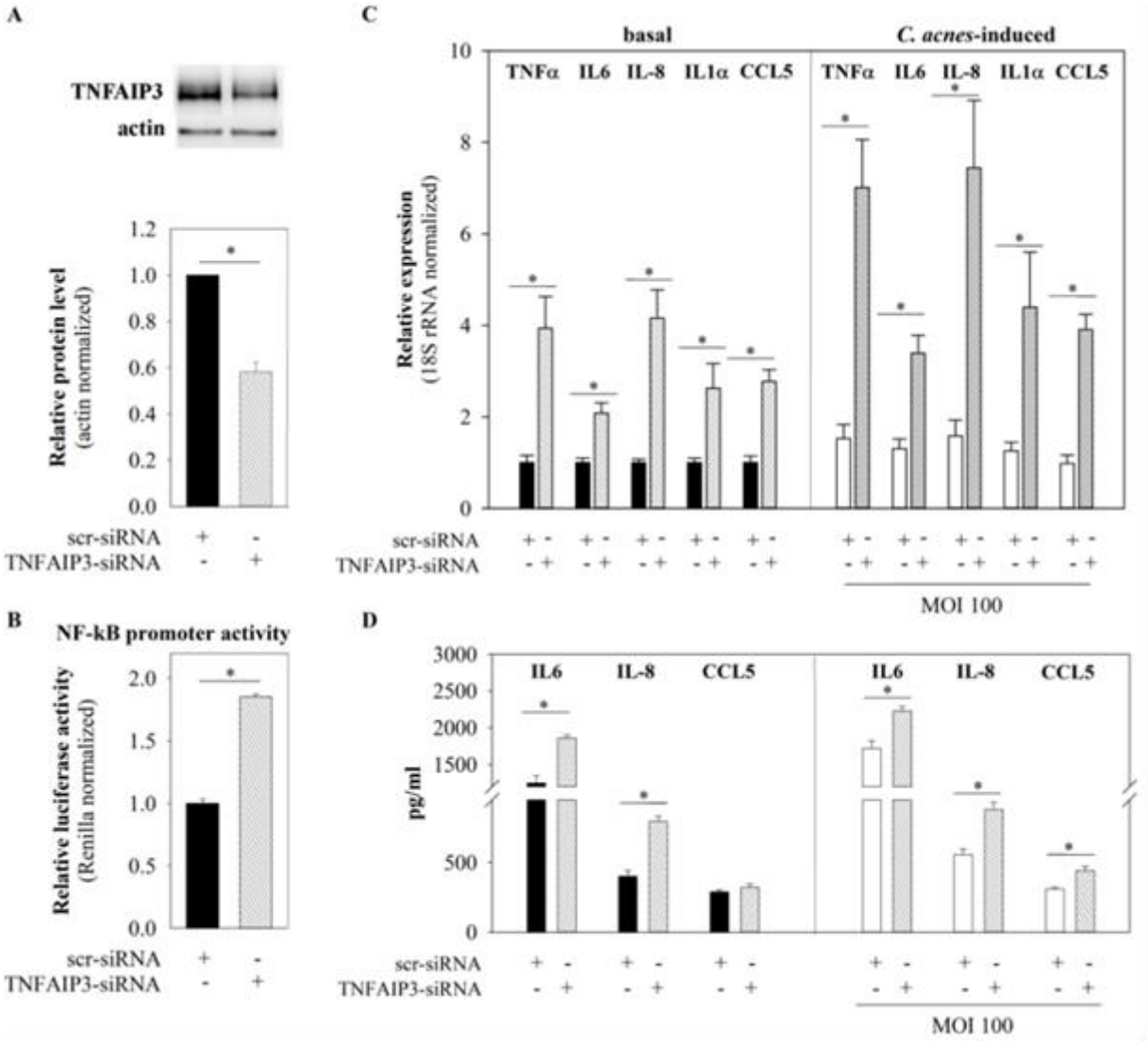

Figure 12. TNFAIP3 negatively regulates downstream targets of the TLR signaling pathways. siRNA-mediated silencing of TNFAIP3 (48 hours) was performed in HPV-KER cells followed by a *C. acnes* (MOI=100) treatment. **(A)** TNFAIP3 protein levels were analyzed by western blotting and quantitated using the Image Pro Plus software. (Representative blot). After 6 hours bacterial treatment, **(B)** NF-κB promoter activities were measured with a luciferase reporter assay, whereas **(C)** mRNA levels of TNFα, IL-1α, IL-6, IL-8, and CCL5 were monitored by real-time RT-PCR. **(D)** After a 24 hours bacterial treatment, secreted cytokine and chemokine levels were measured by ELISA. In luciferase reporter assay all data were normalized to Renilla activity, in western blot analyzes to actin, whereas in real-time RT-PCR analyzes to 18S rRNA, and compared to the time-matched untreated samples transfected with a scrambled siRNA (scr-siRNA). Error bars are SD **(A)** or SE **(B-D).** Statistical analysis: paired, two-sample t-test with Holm–Bonferroni correction: * $p<0.05$.

4.3.4 TNFAIP3 expression differs in acne lesions

According to our current understanding, *C. acnes* plays important roles in the regulation and maintenance of epidermal homeostasis, as well as in acne vulgaris pathogenesis as an opportunistic pathogen because of bacterial dysbiosis. To analyze whether TNFAIP3 tissue levels differ in healthy and lesional skin samples, we analyzed data from a publicly available GEO Profile dataset, comparing the total RNA content of healthy individuals with lesional and non-lesional skin samples of acne patients (56). We found significantly increased levels of TNFAIP3 mRNA expression in the lesional skin samples compared to the corresponding non-lesional skin of acne patients (Figure 13.).

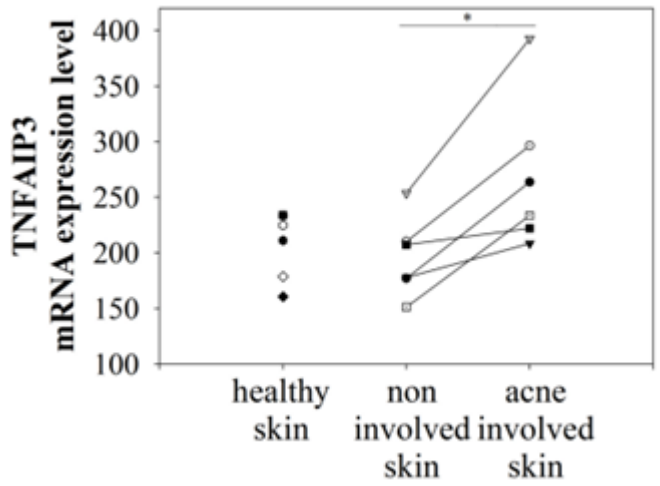

Figure 13. Elevated TNFAIP3 mRNA expression levels are detected in the lesional skin samples of acne patients. TNFAIP3 expression profile from Geo Profile (https://www.ncbi.nlm.nih.gov/geoprofiles/33444972) data were analyzed and compared with paired student t-test with Holm–Bonferroni correction, * $p < 0.05$.

4.4 TNIP1 is expressed in keratinocytes and its expression increases in the presence of *C. acnes*

We also analyzed the role of a known interaction partner of TNFAIP3, TNIP1, in the *C. acnes* induced immune events. We monitored its expression changes in HPV-KER cells upon treatment with the bacterium. We found that TNIP1 is expressed in HPV-KER cells and its mRNA expression rapidly and significantly increased after co-culturing the cells with the *C. acnes* 889 strain (MOI=100), reaching a maximum at 12 to 24 hours (Figure 14/A).

Next, we applied different *C. acnes* strains (889, 6609, ATCC 11828, MOI=100) and compared their effects, but no strain-specific differences were observed: all of the *C. acnes* strains induced similar changes in mRNA expression in HPV-KER cells (Figure 14/B). Subsequently, only the *C. acnes* 889 strain was used in further experiments.

We also found that bacterium-induced changes in TNIP1 expression were dose-dependent: the abundance of mRNA increased in parallel with increasing *C. acnes* 889 bacterial doses (Figure 14/C).

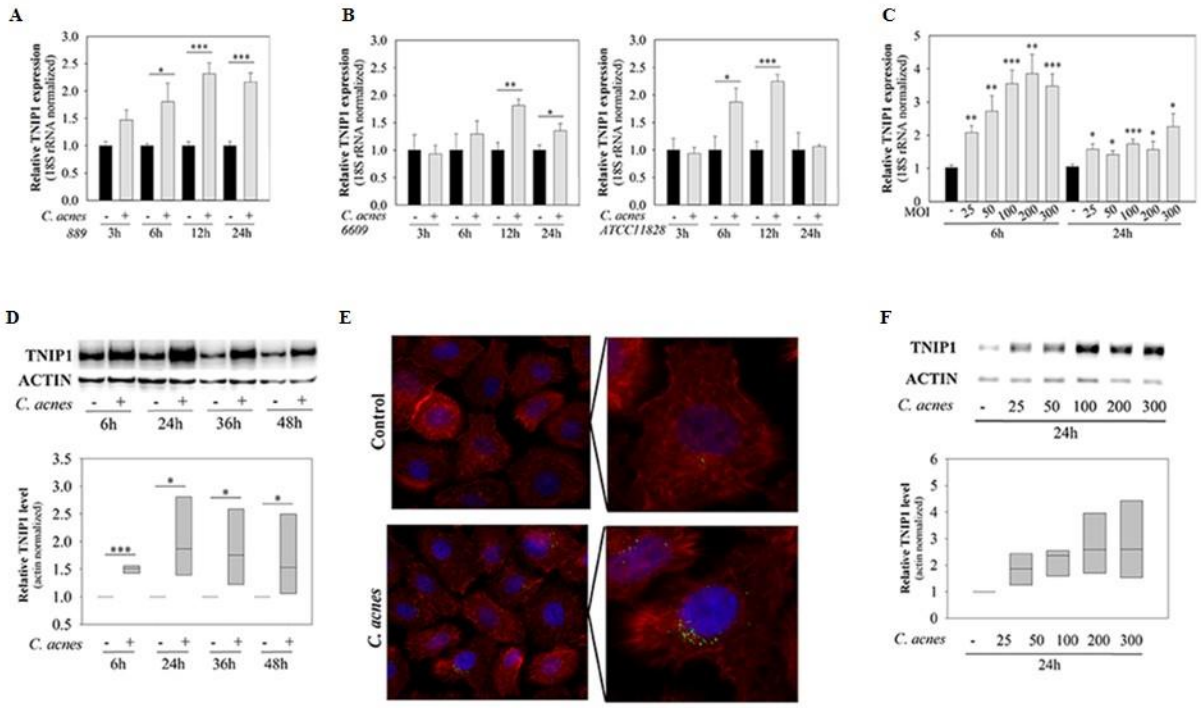

Figure 14. *C. acnes* upregulates TNIP1 mRNA and protein levels in HPV-KER cells. Cells were treated with *C. acnes* 889 (MOI=100, unless otherwise indicated), and changes in mRNA and protein levels were analyzed. **(A-C)** mRNA expression changes were analyzed by real-time RT-PCR, whereas protein expression **(D, F)** was followed by western blot analysis and quantified using Image Pro Plus software or **(E)** was visualized using immunofluorescence staining for TNIP1 (green), DAPI (blue) and Phalloidin (red). All mRNA data were normalized to 18S rRNA, whereas protein levels were normalized to actin and compared to the time-matched untreated control samples. Error bars are SE **(A-C)** or SD **(D, F).** In all panels, statistical analysis: paired, two-sample t-test with Holm–Bonferroni correction: * $p<0.05$.

Next, we analyzed TNIP1 protein levels in HPV-KER cells using western blot analysis and immunocytochemistry. Elevated TNIP1 levels were detected in the 6-hour samples and remained high during the time-course of the experiment (Figure 14/D). Immunocytochemical staining of TNIP1 resulted in the presence of immunofluorescent dots, occurring mostly in the perinuclear region of HPV-KER cells. The number of labeled dots increased after 24 hours of *C. acnes* treatment (MOI=100) (Figure 14/E). Similarly to the mRNA levels, the abundance of TNIP1 protein increased in parallel with increasing *C. acnes* 889 doses (Figure 14/F).

4.4.1 *C. acnes*-induced expression changes of TNIP1 are regulated by different signaling pathways in keratinocytes

To identify which signaling pathways are involved in the regulation of basal and *C. acnes*-induced TNIP1 expression levels in keratinocytes, we treated HPV-KER cells with specific inhibitors of well-known representatives (JNK, NF-κB, p38, ERK1/2, STAT1, and STAT3) of different signaling pathways before the *C. acnes* treatment and analyzed subsequent changes in TNIP1 mRNA expression by real-time RT-PCR. We found that constitutive TNIP1 expression was significantly decreased when JNK and ERK1/2 were inhibited. Furthermore, *C. acnes*-induced changes in TNIP1 expression diminished in response to inhibition of JNK and ERK1/2 as well as of NF-κB and p38. In contrast, no effect was observed with STAT1 and STAT3 inhibition (Figure 15).

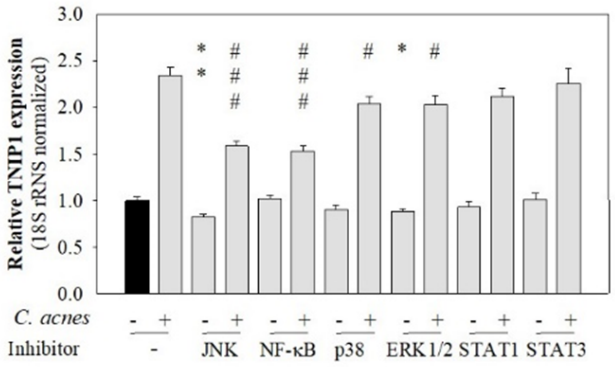

Figure 15. JNK, NF-κB, p38 and ERK1/2 signaling pathways regulate *C. acnes*-induced mRNA levels of TNIP1. HPV-KER cells were pretreated with selective inhibitors for JNK, NF-κB, p38, ERK1/2, STAT1 and STAT3 or, as a control, DMSO for 1 hour. *C. acnes* 889 (MOI=100) challenge was performed for 12 h and TNIP1 mRNA levels were analyzed by real-time RT-PCR and normalized to 18S rRNA. All data were compared to the DMSO-treated control samples. Statistical analysis: paired, two-sample t-test with FDR correction, basal TNIP1 expression *p < 0.05, **p < 0.01 or *C. acnes*-induced TNIP1 expression, #p < 0,05, ###p < 0.001.

4.4.2 Regulation of TNIP1 levels in response to *C. acnes* bacterium is not specific for HPV-KER cells

To confirm that *C. acnes*-induced TNIP1 expression changes were not specific to the HPV-KER cell line, we repeated the co-culturing experiments using NHEK cells and found similar results we observed in case of HPV-KER immortalized keratinocytes. TNIP1 mRNA levels increased in response to *C. acnes* treatment (Figure 16/A), and increased protein levels were also noted in NHEK cell cultures 24 hours after bacterial treatment (Figure 16/B).

We also checked whether the observed results were specific to keratinocyte monolayer cultures, and repeated the experiments using *ex vivo*, full-thickness skin biopsy samples. Six mm punch biopsies of healthy donors were cultured at air–liquid interphase and aliquots of

the *C. acnes* 889 strain were applied to the top (epidermal side) of the samples. We performed an immunofluorescence analysis, which showed that TNIP1 protein was expressed throughout the epidermis and that slightly higher levels were detected in the less differentiated, basal layers. Within the keratinocytes, localization was primarily cytoplasmic and perinuclear. Elevated TNIP1-staining levels were found 24 hours after *C. acnes* treatment (Figure 16/C).

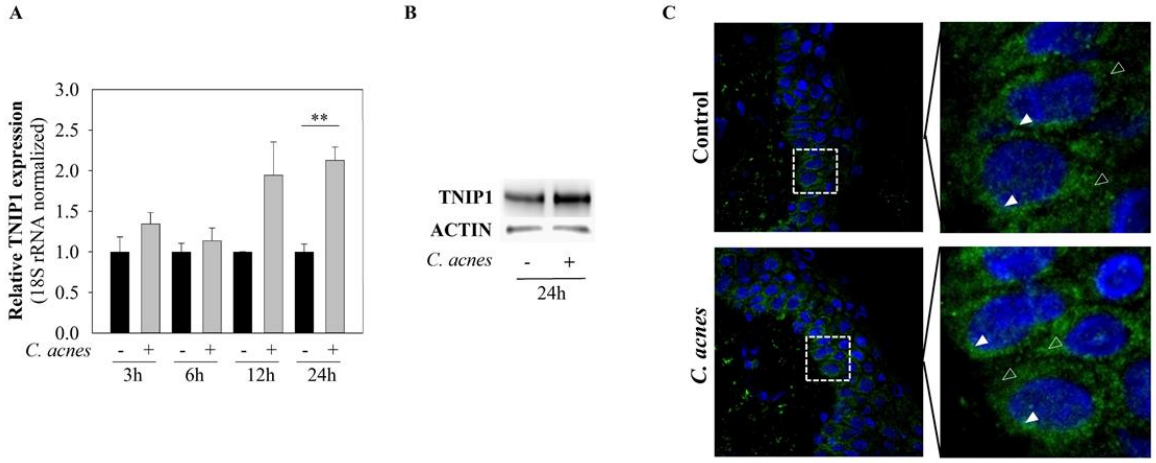

Figure 16. TNIP1 mRNA and protein expression increases in response to *C. acnes* treatment in NHEK cells and ex *vivo* OS models. NHEK cells were treated with the *C. acnes* 889 strain (MOI=100) and **(A)** TNIP1 mRNA expression changes of were analyzed by real-time RT-PCR, whereas protein expression **(B)** was followed by western blot analysis. mRNA data were normalized to 18S rRNA and compared to time-matched untreated samples. **(C)** OS samples were treated with 3×10^7 bacterium/cm^2 for 24 hours, and cells were visualized using immunofluorescence staining for TNIP1 (green) and DAPI (blue). Empty arrows indicate cytoplasmic location and filled arrows indicated perinuclear localization. Statistical analysis: paired, two-sample t-test with FDR correction: $^{**}p < 0.01$.

4.4.3 TNIP1 down-regulates both constitutive and *C. acnes*-induced inflammatory cytokines and chemokines expression

To analyze the role of TNIP1 in the regulation of *C. acnes*-induced inflammatory events, we experimentally modified endogenous TNIP1 levels with cDNA-based transient overexpression or siRNA-mediated silencing. We monitored the expression of selected pro-inflammatory cytokines and chemokines that are known downstream targets of the TLR signaling pathway, as well as the promoter activity of the NF-κB transcription factor, using real-time RT-PCR, ELISA analysis and a luciferase- reporter assay.

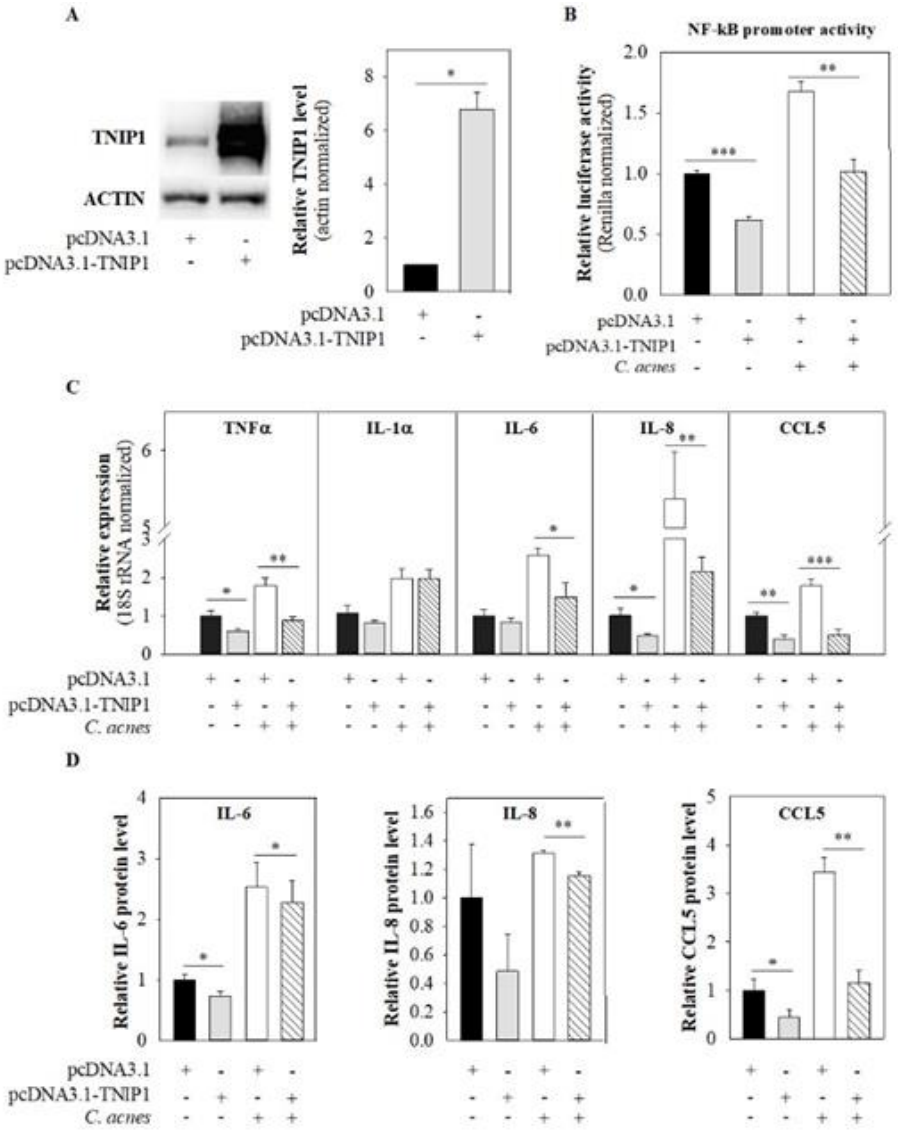

Figure 17. TNIP1 overexpression affects the downstream targets of the TLR signaling pathway. TNIP1 overexpression (24 hours) was performed and HPV-KER cells were treated with *C. acnes* (MOI=100). **(A)** TNIP1 protein levels were analyzed by western blot and quantitated using the Image Pro Plus software. (Representative blot.) After 6 hours bacterial treatment, **(B)** NF-κB promoter activities were measured with luciferase reporter assay, whereas **(C)** mRNA levels of TNFα, IL-6, IL-8 and CCL5 were monitored by real-time RT-PCR. **(D)** After 24 hours bacterial treatment, secreted cytokine and chemokine levels were measured by ELISA and relative protein levels are presented. In luciferase reporter assay all data were normalized to Renilla , in western blot analyzes to actin, whereas in real-time RT-PCR analyzes to 18S rRNA and compared to the time-matched untreated samples transfected with an empty vector (pcDNA3.1). Statistical analysis: paired, two-sample t-test: * $p< 0.05$, ** $p<0.01$, ***$p<0,001$.

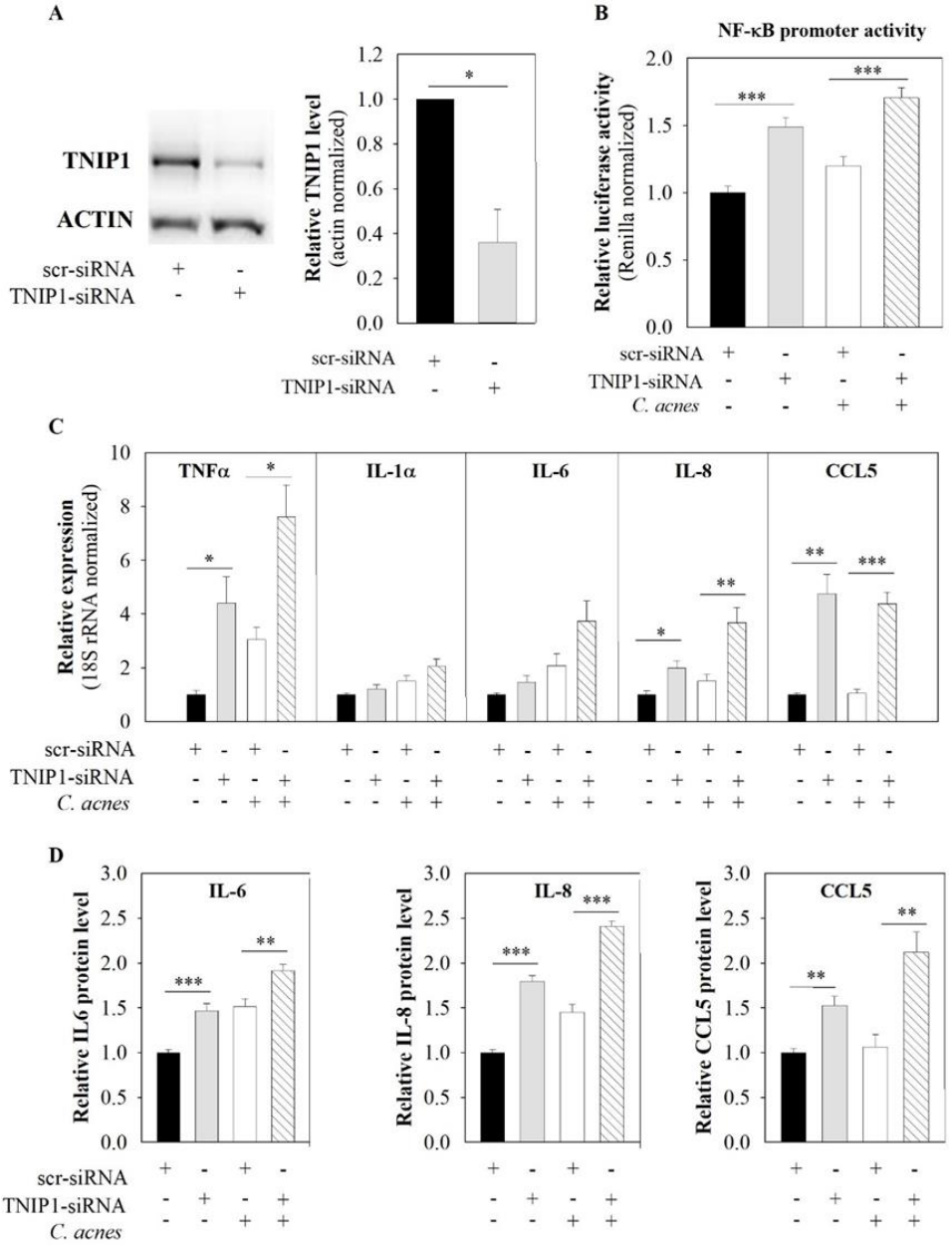

Figure 18. TNIP1 knockdown affects the downstream targets of the TLR signaling pathway. TNIP1 siRNA-mediated silencing (48 hours) was performed and HPV-KER cells were treated with *C. acnes* (MOI=100). **(A)** TNIP1 protein levels were analyzed by western blot and quantitated using the Image Pro Plus software. (Representative blot.) After 6 hours bacterial treatment, **(B)** NF-κB promoter activities were measured with a luciferase reporter assay, whereas **(C)** mRNA levels of TNFα, IL-6, IL-8 and CCL5 were monitored by real-time RT-PCR. **(D)** After 24 hours bacterial treatment, secreted cytokine and chemokine levels were measured by ELISA and relative protein levels are presented. In luciferase reporter assay all data were normalized to Renilla, in western blot analyzes to actin, whereas in real-time RT-PCR analyzes to 18S rRNA and compared to the time-matched untreated samples transfected with scrambled siRNA (scr-siRNA). Statistical analysis: paired, two-sample t-test: * $p< 0.05$, ** $p<0.01$, ***$p<0,001$.

cDNA-based transient overexpression resulted in markedly increased TNIP1 protein levels in HPV-KER cells (Figure 17/A). As a consequence, significantly decreased constitutive and *C. acnes*-induced NF-κB promoter activities were measured in the subsequent luciferase reporter assay (Figure 17/B). mRNA expression of constitutive and *C. acnes*-induced TNFα, IL-8 and CCL5 also decreased compared to the empty-vector transfected samples. Besides, bacterium-induced mRNA levels of IL-6 decreased, whereas IL-1α levels were not affected (Figure 17/C). Overexpression of TNIP1 also decreased IL-6, IL-8 and CCL5 protein secretion (Figure 17/D).

In contrast, siRNA-mediated silencing of TNIP1 led to markedly decreased TNIP1 protein levels (Figure 18/A). As a consequence, significantly increased constitutive and bacterial-induced NF-κB promoter activities were found in a luciferase reporter assay (Figure 18/B). Constitutive and *C. acnes*-induced expression of TNFα, IL-8, and CCL5 mRNAs markedly increased, whereas IL-1α and IL-6 expression increased moderately in response to TNIP1 silencing (Figure 18/C). Constitutive and bacterium-induced secretion of IL-8, IL-6, and CCL5 were also elevated in TNIP1 silenced HPV-KER cells (Figure 18/D).

4.4.4 ATRA induces TNIP1 expression and affects the levels of downstream targets of the TLR signaling pathway

The TNIP1 promoter contains retinoic acid response elements (RARE) and retinoic-acid-receptor binding sites. These elements are involved in the induction of *TNIP1* expression by retinoic acid under permissive epigenetic conditions in different cell lines (60). Since ATRA is an effective drug used for acne treatment, we examined whether this compound is capable of regulating TNIP1 and, thus, the expression of downstream targets of the TLR signaling pathway in keratinocytes.

We observed that ATRA treatment led to slightly elevated TNIP1 mRNA levels, although this effect was not statistically significant (Figure 19/A), and to significantly increased TNIP1 protein expression (Figure 19/B). In addition, constitutive and *C. acnes*-induced mRNA expression of TLR-2 and the pro-inflammatory TNFα and CCL5 decreased upon ATRA treatment. In contrast, both constitutive and *C. acnes*-induced TLR-4 and IL-8 mRNA expression levels increased, whereas TLR3 and IL-6 mRNA levels were not affected by the drug (Figure 19/D, E).

To confirm that the effects of ATRA on TNIP1 expression were not specific to the HPV-KER cell line, we also applied the drug to the upper, epidermal part of OS models. We found that TNIP1 protein expression levels had increased in all epidermal layers 24 hours after ATRA treatment (Figure 19/C), in a manner similar to observed for immortalized keratinocytes.

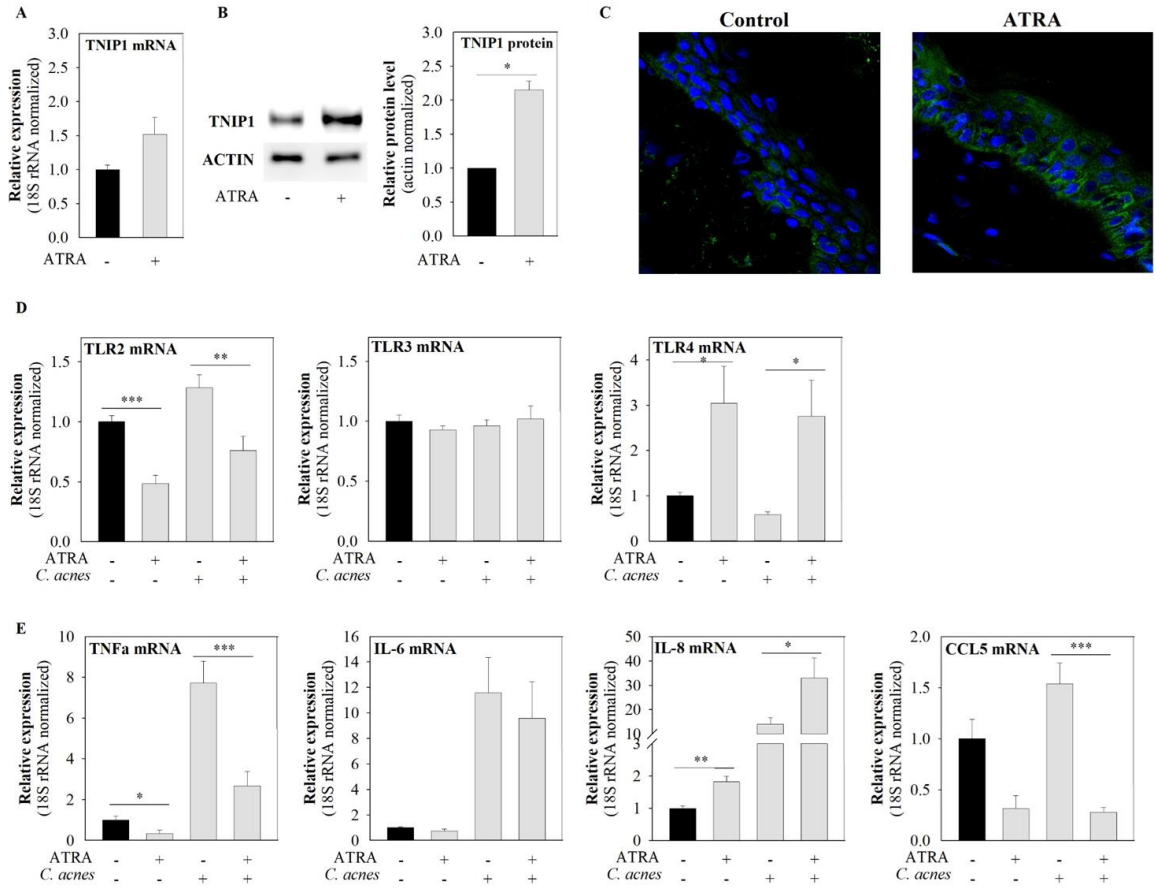

Figure 19. ATRA induces TNIP1 expression and affects the expression of TLRs and downstream targets of the TLR signaling pathways. HPV-KER cells **(A, B)** were treated with 10^{-6} M ATRA for 48 hours or the OS cultures **(C)** with 1.5×10^{-6} M for 24 hours. TNIP1 mRNA levels were analyzed by real-time RT-PCR, whereas protein levels were followed by western blot analyzes and quantitated using the Image Pro Plus software or visualized by immunofluorescence staining for TNIP1 (green) and DAPI (blue). Subsequently, HPV-KER cells were challenged with *C. acnes* (MOI=100) for 6 h and the mRNA expression changes of TLRs **(D)** and selected pro-inflammatory molecules was analyzed by real-time RT-PCR. mRNA data were normalized to 18S rRNA, protein levels were normalized to actin and compared to time-matched untreated samples. Errors are SD **(B)** or SE **(A, D, E)**. Statistical analysis: paired, two-sample t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5 Discussion

The human skin harbors a specialized microbiota that protects against pathogens, helps to maintain proper skin functions, and also serves as a part of the immunological barrier. These are not specific functions of the cutaneous microbial community, similar functions have also been identified for the gut microbiota (61). One important feature of the resident skin community is that its members keep the cutaneous immune system at a basal activated state. Such low levels of TLR activation may contribute to the maintenance of basal expression levels of different genes (*e.g.*, cytokines, chemokines, and inducible antimicrobial peptides), playing important roles in the formation and maintenance of the immunological barrier (62). In healthy skin, these events do not lead to inflammation. However, under certain conditions, different commensal microbes may be involved in inflammatory skin diseases, such as *C. acnes* in acne pathogenesis (30,63). The mechanisms underlying this duality – the protective and pathogenic aspects of the continual presence of the skin microbiota – are still unclear (61,64).

C. acnes, a dominant member of the human skin microbiome, is recognized by TLR2 and TLR4 in epidermal keratinocytes. This bacterium can induce innate immune and inflammatory events, such as the production of inflammatory cytokines, chemokines, antimicrobial peptides, and other inflammatory mediators, as well as autophagy in keratinocytes, and, through this induction, plays a role in the pathogenesis of acne vulgaris (21,22,63,65). However, at the beginning of early adolescence, this bacterium becomes overwhelmingly dominant in the skin, yet does not induce macroscopically detectable inflammation in the majority of the PSUs (8,11). These observations suggest the existence of regulatory mechanisms that control *C. acnes*-induced immune activation.

In the past decades, several negative regulators of the TLR and the NF-κB signaling pathways have been identified. These play important roles in the protection against exaggerated immune activation. Since their discovery, an increasing number of publications have reported a correlation between genetic variations of these proteins and altered expression and/or dysfunctional proteins, as well as with diseases associated with inflammation, such as psoriasis, systemic lupus erythematosus, pneumonia and cancer (34,66–69). Taken together, these results suggest that proper regulation and function of these factors are indispensable for

the maintenance of health, but the role of these molecules in the assuagement of microbiome-induced immune activation has been poorly investigated.

In our studies, we aimed to investigate whether well-known negative regulators of TLR signaling pathways, SIGIRR, TOLLIP, TNFAIP3 and TNIP1, have any role in the *C. acnes*-induced innate immune activation of keratinocytes. For that, we systematically analyzed their expression changes upon *C. acnes* challenge and their role in the bacterium-induced innate immune events using *in vitro* and *ex vivo* model systems.

SIGIRR is widely expressed in several cell types, among others, in cells that have direct contact with the human microbiota, including lung and intestinal epithelial cells (IECs) (70). In most cases, TLR stimulation by pathogens or synthetic TLR ligands dampens SIGIRR levels (40). We found that SIGIRR is also expressed in immortalized keratinocytes, but none of the *C. acnes* strains we used in our studies affected its expression in HPV-KER cells.

Many studies also showed, that decreased SIGIRR levels contributed to local and systemic inflammation upon pathogen infection in SIGIRR-deficient mice models, and enhanced inflammatory mediator production upon TLR ligand stimuli in SIGIRR-silenced intestinal epithelial cells (40,71). In contrast, SIGIRR did not suppress TLR signaling in intra-renal and tubular epithelial cells, suggesting a cell-type specific role of this molecule (72,73). In our experiments, transient, siRNA-mediated silencing of SIGIRR did not affect basal and *C. acnes*-induced TNFα mRNA expression. These results suggest, that even though SIGIRR is expressed in HPV-KER cells, it does not seem to play important roles in the control of *C. acnes*-induced signaling cascades in keratinocytes.

TOLLIP is also present in different microbiota-colonized surfaces, and elevated expression levels were found in response to different TLR ligands in IECs (74,75). We found that TOLLIP is expressed in human, *in vitro* cultured keratinocytes, but its mRNA and protein levels remained unchanged upon *C. acnes* challenge.

Altered TOLLIP levels can influence the outcome of TLR2 and TLR4 activation, and inflammatory mediator production in IECs (75), and TOLLIP deficient mice were susceptible to colitis (76,77). We experimentally modified TOLLIP levels by siRNA-mediated silencing and found that in the silenced cells, the basal and *C. acnes*-induced TNFα levels remained unchanged. These results suggest that TOLLIP is not a major regulator of the bacterium-induced immune activation in keratinocytes.

We also investigated the role of TNFAIP3 in the *C. acnes*-induced immune processes. In agreement with other studies (48), we showed that TNFAIP3 is expressed through the entire epidermis, exhibiting higher levels in the more differentiated keratinocyte layers. This gradient may be formed parallel with the natural differentiation processes, as one of the major regulators of keratinocyte differentiation is calcium (Ca^{2+}), and together with Ca^{2+}-induced differentiation, TNFAIP3 expression also increases in normal human *in vitro* cultured keratinocytes (48,78). The molecular details of these events are currently not known, but it may be mediated through the DREAM transcription factor, which is Ca^{2+} regulated and also plays important roles in the regulation of TNFAIP3 transcription(58,79).

We also analyzed whether *C. acnes* influenced TNFAIP3 expression levels *in vitro* and *ex vivo*. We found that both the mRNA and protein expression rapidly increased in a dose-dependent manner in response to the bacterium in cultured HPV-KER cells, as well as in the epidermal part of an *ex vivo* skin model. A similar effect was described recently for another member of the skin microbiota, *S. epidermidis* (49). These results, taken together, indicate that the TNFAIP3 protein may play important roles in the regulation of immune and inflammation activation in response to various members of the cutaneous microbiota.

According to our current knowledge, *C. acnes* plays an important role in the pathogenesis of acne vulgaris, as a result of microbial dysbiosis (11). We were curious to see whether TNFAIP3 levels differ in control individuals and in the skin samples from acne patients. To answer this question, we compared publicly available microarray data in the GEO Profile database (ID: 33444972) and found that in patients, mRNA expression of TNFAIP3 significantly increases in papules compared to the non-lesional skin samples of the same individuals. In contrast to that, no major differences were noted comparing the healthy controls and the normal-looking skin of patients. These results suggest that, in the lesional skin, apart from the production of different pro-inflammatory mediators, the expression of anti-inflammatory molecules also increases. These latter molecules may play important roles in inflammation control to avoid excessive, potentially tissue-damaging reactions. To determine whether increased TNFAIP3 mRNA levels in the lesional skin corresponds to elevated protein levels, further research is necessary. If mRNA and protein levels correspond, the accumulation may be an important step during the healing of lesions and the restoration of epidermal homeostasis.

We also analyzed whether TNFAIP3 expression differs in response to different *C. acnes* strains. We found no major differences in the TNFAIP3 mRNA expression when the HPV-KER cells were co-cultured with *C. acnes* 889, ATCC11828 and 6609 bacteria strain.

Multiple signaling pathways and transcriptional factors are involved in the transcriptional regulation of TNFAIP3. In LPS-activated bone-marrow-derived macrophages from mice, NF-κB and p38 regulate TNFAIP3 expression through its NF-κB and C/EBPB binding sites (59). However, in Pam3CSK4-activated THP1 cells, NF-κB inhibition has no effect: GSK3-related pathways regulate changes in TNFAIP3 expression, possibly through the cAMP response element-binding protein (CREB) (80). Others also showed that the DREAM complex represses its transcriptional activation through downstream-responsive elements (DRE3 and DRE4) binding sites in mice and HLMVECs (58). These findings suggest that the transcriptional regulation of TNFAIP3 is possibly dependent on the type of cell and stimuli. We found that the basal mRNA expression of TNFAIP3 is regulated by JNK, whereas JNK and NF-κB signaling pathways are involved in the control of *C. acnes*-induced changes in TNFAIP3 mRNA and protein expression, for which p38 and ERK1/2 inhibition had no effect. Dual inhibition of JNK and NF-κB signaling pathways almost completely inhibited *C. acnes*-induced changes in mRNA expression, which suggest that these two signaling pathways have a major impact on the bacterium-induced regulation of TNFAIP3 mRNA levels in HPV-KER cells.

Based on our findings, TNFAIP3 possibly operates in a negative regulatory cycle in keratinocytes. In this control loop, TNFAIP3 regulates the signaling consequences of *C. acnes*, which, in turn, also affects TNFAIP3 levels (Figure 20).

TNFAIP3 may help *S. epidermidis* to persist as a commensal on the human skin, through the control of bacterium-induced NF-κB activation, IL-1β and hBD2 production in keratinocytes (49). Based on these findings, we aimed to analyze whether this negative regulatory function also occurs in *C. acnes*-induced inflammatory processes. For these experiments, we found that silencing TNFAIP3 significantly increased basal NF-κB promoter activities, as well as the mRNA expression of pro-inflammatory cytokines and chemokines (TNFα, IL-1α, IL-6, IL-8 and CCL5) in HPV-KER cells. Co-culturing of silenced cells with the *C. acnes* 889 strain also lead to increased TNFα, IL-1α, IL-6, IL-8 and CCL5 mRNA expression, as well as elevated IL-8, IL-6 and CCL5 protein secretions. These data argue that

TNFAIP3 plays important roles in the regulation of *C. acnes*-driven molecular events in keratinocytes as well as in the maintenance of cellular homeostasis by regulating the cutaneous inflammatory program under homeostatic conditions (81).

Apart from the control of the microbiota-induced processes, studies suggests that TNFAIP3 functions in, for example, poly-I:C-induced inflammatory responses in human keratinocytes, as well as in imiquimod- and acetone-induced psoriasis (PSO) and dermatitis in mouse models, respectively (47,48). TNFAIP3 also plays important roles in the induction of Pam3CSK4-tolerance in monocytic THP-1 cells, arguing for a generalized role for this molecule in different TLR-mediated molecular processes (80).

Based on the analysis of publicly available data, we could show that TNFAIP3 levels differ in the non-lesional and lesional skin samples of acne patients. Interestingly, acne is not the only inflammatory skin disease in which TNFAIP3 levels change. In psoriasis and atopic dermatitis (AD), studies showed lower TNFAIP3 mRNA levels in lesional skin samples, compared to the skin from healthy individuals. Moreover, in PSO patients, TNFAIP3 mRNA and protein levels were lower in lesional skin than in non-lesional samples of the same patients. In contrast, TNFAIP3 mRNA expression levels in AD patients did not differ in the involved and non-involved samples (47,48). It is not clear whether this observation represents a true difference in the pathogenesis of these chronic inflammatory skin conditions. One possible explanation is that the levels of negative regulators may rapidly change throughout the different phases of disease pathogenesis, and the above observations represent only temporal variations. It is interesting to note, however, that inability to control innate immune activation due to decreased TNFAIP3 levels may lead to persisting, chronic inflammation, and the net outcome and the severity of inflammation possibly depends on the balance of pro- and anti-inflammatory factors. As a result, this molecule may serve in the future as a drug target for treatment.

Last, but not least we also investigated the role of TNIP1, and we found that it is also a possible negative regulator of *C. acnes*-induced inflammatory events in HPV-KER cells. It is expressed in cultured keratinocytes, and its mRNA and protein expression increased in response to *C. acnes* treatment; and the extent of activation depended on the bacterial dose. These changes were not specific for the HPV-KER cell line, as similar results were obtained using NHEK cells. In *ex vivo* OS models we found that TNIP1 was expressed in all epidermal

layers, which was consistent with previous studies (52). Some differences were also noted, as we detected mostly cytoplasmic and perinuclear staining within the keratinocytes, in contrast to previous studies, where cytoplasmic and also intensive nuclear staining of TNIP1 was observed in HaCaT cells (52,82). The reason for these differences is not clear but may be caused by the differences in the properties of the used models, or the dissimilarities of the applied experimental conditions. When we analyzed the effect of the *C. acnes* treatment, we found elevated TNIP1 levels in the epidermis of the OS cultures. These results are in agreement with our findings using NHEK and HPV-KER cells, showing that, in addition to pro-inflammatory mediators, the bacterium also induces the expression of anti-inflammatory factors, possibly to avoid excessive inflammation and immune activation. Our results strongly suggest that TNIP1 may be one of the negative regulators of *C. acnes*-induced molecular events. These effects were independent of the used *C. acnes* strains, we did not observe any differences when we co-cultured our models with phylogenetically different strains. This was similar to what we observed in the case of TNFAIP3. These findings suggest that strain-specific biological effects of *C. acnes* are likely not due to the ability of differential induction of various negative regulators upon immune and inflammation induction in keratinocytes.

Next, were interested in the signaling pathways and regulatory molecules that may regulate TNIP1 levels under basal conditions and upon *C. acnes* induction. *In silico* analyzes predicted the presence of putative binding sites for NF-κB, AP-1, SP1, SP3 and C/EBPB in the TNIP1 promoter region (59,83). Gene expression and ChIP assays have confirmed that NF-κB and SP1 binding sites are active in HeLa cells (82,84). Our finding that specific JNK, NF-κB, p38 and ERK1/2 inhibitors decreased the bacterium-induced upregulation of TNIP1 suggest that these signaling pathways may be involved in the regulation of *C. acnes*-induced TNIP1 expression in keratinocytes. Moreover, the intricate TNIP1 regulation pattern also suggests that this molecule may have important roles in different processes.

To confirm the negative regulatory role of TNIP1 in *C. acnes*-induced molecular events, we modified its endogenous levels by cDNA-based overexpression and siRNA-mediated silencing and examined the expression of downstream elements of TLR signaling pathways. NF-κB, one of the main mediators of signaling cascades activated in our experiments, is induced upon exposure to bacteria (21,27). Our results, that TNIP1 overexpression decreased basal and *C. acnes*-induced NF-κB promoter activities and mRNA levels of TNFα, IL-8, and

CCL5 as well as secretion of IL-8, IL-6 and CCL5 correlated well with findings on other cell types. In HeLa cells, TNIP1 overexpression inhibited constitutive TNFα, IL-1α expression and lipopolysaccharide (LPS)-induced activation of NF-κB (50,85).

In TNIP1-silenced cells, we observed increased constitutive NF-κB promoter activities and elevated pro-inflammatory cytokine and chemokine mRNA and protein levels. TNIP1 silencing also increased *C. acnes*-induced NF-κB promoter activity, mRNA levels and secretion of the mediators mentioned above. These results further support the conclusion that TNIP1 plays a role in the regulation of *C. acnes*-induced events in HPV-KER cells and possibly in the maintenance of homeostatic conditions. In a recent report, the authors have also demonstrated that TNIP1-silenced HaCaT cells were hypersensitive to synthetic TLR3 and TLR2/6 ligands and subsequently increased JNK and p38 phosphorylation and nuclear translocation of NF-κB. They also observed increased levels of secreted IL-6 and IL-8 compared to control cells (53). Overall, these data indicate a negative regulatory role of TNIP1 in TLR signaling events in keratinocytes.

Other reports have also shown the importance of TNIP1 as a gatekeeper in NF-κB, JNK, and p38 mediated processes, including the prevention of fetal liver apoptosis in a murine model and TNFα-induced apoptosis in different cell lines (86,87). In contrast, although TNIP1-deficient mice develop a progressive, lupus-like inflammatory disease, isolated TNIP1-deficient macrophages and dendritic cells showing no differences in pro-inflammatory signaling pathways with respect to IκBα degradation and resynthesis and phosphorylation of different MAPKs (p38, ERK1/2, and JNK1/2) compared to wild type mice upon CpG-DNA or TLR4 (LPS) stimulation (88,89). These findings suggest that the role of TNIP1 during TLR activation might be cell-type specific and depends on the nature of the stimuli.

Earlier studies found functional RARE elements in the TNIP1 promoter region, and that TNIP1 was induced by ATRA in HeLa cells under permissive epigenetic conditions, in the presence of Trichostatin A co-treatment (60). Trichostatin A inhibits histone deacetylase I and II and alters gene expression by opening chromatin and allowing transcription factors to bind, thus, promoting transcription of different genes. We applied ATRA, an active form of retinoic acid, to HPV-KER cells and found that TNIP1 mRNA and protein expression levels increased in response to the treatment without the addition of chromatin-modification agents. Furthermore, treatment of OS models led to similar results throughout the entire epidermis,

suggesting that these effects were not specific to HPV-KER cells. It is not currently clear why HeLa cells behaved differently; however, differences in the duration of ATRA exposure or in the responsiveness of the different cells used might be responsible.

Despite the fact that retinoids, including ATRA, are widely used as an effective drug for acne therapy, the exact mechanism of action is not completely understood. Retinoids have been shown to promote cell proliferation, inhibit keratinocyte terminal differentiation, decrease the size of sebaceous glands and, indirectly, reduce the amount of *C. acnes* (90). Studies of the effect of retinoids on innate immunity are limited, and the results are often dependent on the cells used (60,91–94). In most cases, ATRA application to monocytes and macrophages decreased TLR2 abundance as well as the expression of selected pro-inflammatory mediators; however, the mechanism of these effects remains unclear (93,95,96).

In our experiments, ATRA treatment decreased the level of constitutive and *C. acnes*-induced pro-inflammatory mediators TNFα and CCL5 but increased IL-8 levels. These results are consistent with the findings of others; similar IL-8 expression changes were observed in NHEK and also in other cell types in response to ATRA (91,92,97). In NHEK cells, NF-κB and p38 signaling might contribute to these events (91).

Based on all these data, we conclude that ATRA may regulate TNIP1 expression and, as a consequence, negatively affect *C. acnes*-induced inflammatory events in keratinocytes. Our proposed model may offer a possible, novel mode of retinoid action in acne treatment.

TLR2 expression is increased in the epidermis and monocytes isolated from acne patients compared to healthy controls, and this increase might be a result of *C. acnes*-induced inflammatory events. ATRA treatment decreased TLR2 levels in monocytes isolated from both healthy donors and acne patients following isotretinoin therapy, although TLR4 expression was not affected (21,95,96,98,99). We found similar changes in expression for TLR2 (decreased) and TLR4 (increased) in HPV-KER cells, suggesting that ATRA has opposite effects on these two receptors in keratinocytes.

We propose that the increased bacterial load in acne-prone follicles is a factor leading to the formation of inflammatory symptoms. Retinoids may attenuate *C. acnes*-induced inflammation by decreasing the sizes of sebaceous glands and sebum secretion and, subsequently, control the *C. acnes* load. In addition, TLR2 levels are also attenuated, preventing the sensing of this Gram-positive bacterium, which leads to deleterious

inflammation. By decreasing the expression of TLR2, an important gatekeeper of the skin, the risk of opportunistic infections is increased. To modulate this effect, expression of another gatekeeper, TLR4 is elevated. Thus, multiple levels of sensing and signal transduction may be available in the defense against potentially harmful microbial invaders. The fact that opportunistic bacterial and fungal infections are rare in the lesional skin of acne patients is consistent with our hypothesis.

TNIP1 may regulate *C. acnes*-induced signaling events through the establishment of a negative-regulatory feedback loop controlling NF-κB activity in keratinocytes, similarly to TNFAIP3. Other signaling pathways, such as nuclear-receptor signaling cascades that are activated by retinoids, may also affect TNIP1 levels, which in turn can modify the outcome of the induced processes (Figure 20).

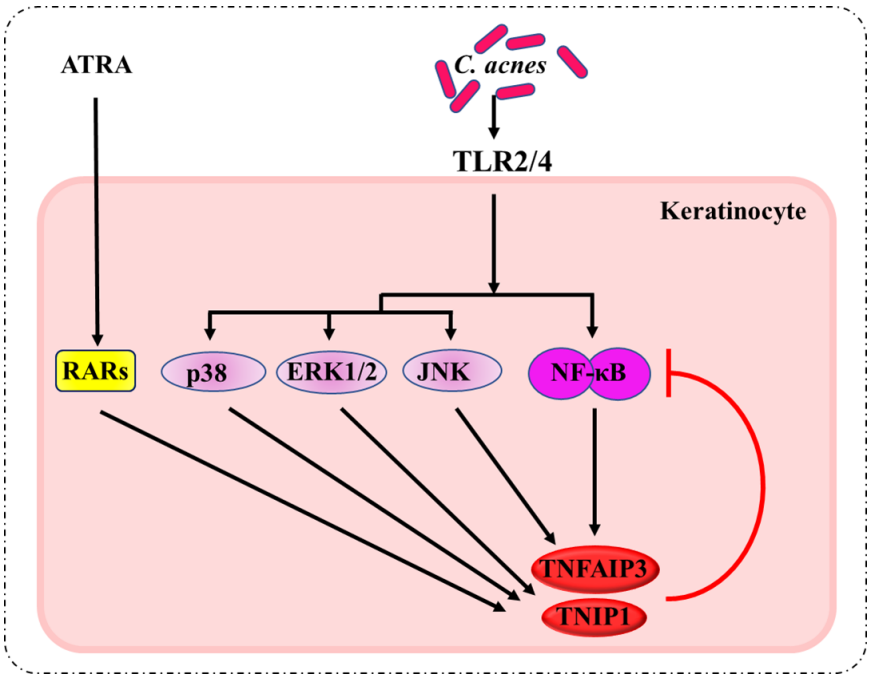

Figure 20. Negative regulation of *C. acnes*-induced immune activation in keratinocytes by TNFAIP3 and TNIP1.

In summary, TNFAIP3 and TNIP1 may function as negative regulators in keratinocytes that control *C. acnes*-induced inflammatory events, playing important roles in maintaining the homeostasis between the skin cells and the cutaneous microbiome. Development of novel, well-tolerated, TNFAIP3 and TNIP1-specific acne therapeutic modalities could potentially reduce inflammation without the harmful side effects of currently available treatment options (antibiotic, retinoid or hormonal formulation usage).

6 Summary

Human skin cells recognize the presence of the skin microbiome through pathogen recognition receptors. Epidermal keratinocytes are known to activate TLR2 and TLR4 response to the commensal *C. acnes* bacterium, and subsequently induce innate immune and inflammatory events, which contributes to the pathogenesis of acne vulgaris. Healthy skin does not exhibit inflammation or skin lesions, even in the continuous presence of the same microbes. As the molecular mechanism for this duality is still unclear, we aimed to identify factors and mechanisms that control the innate immune response to *C. acnes* in keratinocytes.

We found that the expression of the well-known negative regulators of the TLR signaling cascade, TNIP1 and TNFAIP3, were rapidly induced in response to *C. acnes* in human, *in vitro* cultured keratinocytes as well as in OS models. Expression changes were dependent on the applied *C. acnes* dose, but not on the used bacterium strain. Bacterial-induced changes in TNIP1 expression were regulated by signaling pathways involving NF-κB, p38, ERK1/2, and JNK, whereas TNFAIP3 expression were regulated by JNK and NF-κB pathways. Experimental modification of TNIP1 and TNFAIP3 levels affected the activity of NF-κB transcription factor and subsequent inflammatory cytokine and chemokine mRNA and protein levels. We found that all-trans retinoic acid (ATRA) induced elevated TNIP1 expression in HPV-KER cells and also in OS models, where TNIP1 levels increased throughout the epidermis. Based on these findings, we propose that ATRA may exhibit dual effects in acne therapy by both affecting the expression of the negative regulator TNIP1, and attenuating TLR2-induced inflammation.

In summary, TNFAIP3 and TNIP1 play important roles in the regulation of TLR-induced signaling pathways in keratinocytes, and, through these roles, contribute to the control of *C. acnes*-induced innate immune and inflammatory events (Figure 19). By fine-tuning the microbiota-induced signaling processes, these two molecules may help to maintain the homeostatic conditions between the skin and the cutaneous microflora.

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