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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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Publications related to the subject of the thesis

I. **Lilla Erdei**, Beáta Szilvia Bolla, Renáta Bozó, Gábor Tax, Edit Urbán, Lajos Kemény, Kornélia Szabó. TNIP1 regulates *Cutibacterium acnes*-induced innate immune functions in epidermal keratinocytes. Front Immunol. 2018 Sep 24;9:2155. doi: 10.3389/fimmu.2018.02155.
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II. K. Szabó, **L. Erdei**, B. Sz. Bolla, G. Tax, T. Bíró, L. Kemény. Factors shaping the composition of the cutaneous microbiota. Br J Dermatol. 2017 Feb;176(2):344-351
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III. Lilla Erdei, Beáta Szilvia Bolla, Renáta Bozó, Gábor Tax, Edit Urbán, Katalin Burián, Lajos Kemény, Kornélia Szabó. TNFAIP3 negatively regulates *Cutibacterium acnes*-induced innate immune events in epidermal keratinocytes. (Accepted for publication. "Published in Acta Dermato-Venereologica by Society for Publication of Acta Dermato-Venereologica" https://www.medicaljournals.se/acta/content/abstract/10.2340/00015555-3707)

Other publications

IV. Szabó, K; Bolla, BSz; **Erdei, L**; Kemény, L. A bőrünkön élő mikrobák szerepe az egészséges bőrben és az acne vulgaris kialakulása során. ORVOSTOVÁBBKÉPZŐ SZEMLE 24 : 12 pp. 26-30. , 5 p. (2017)

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VI. Bolla BS, **Erdei L**, Urbán E, Burián K, Kemény L, Szabó K. Cutibacterium acnes regulates the epidermal barrier properties of HPV-KER human immortalized keratinocyte cultures. Sci Rep (2020) 10: doi:10.1038/s41598-020-69677-6.

Introduction

The human skin harbors a specialized microbiota which populates the epidermis, and the specialized anatomical and functional organelles called pilosebaceous units (PSU). 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. Acne vulgaris is one such a disease, wherein the role of *Cutibacterium acnes* (*C. acnes*) has been investigated for a long time.

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. They can sense the presence of these microbes through their pathogen recognition receptors, *e.g.*, Toll-like receptors (TLRs) and subsequently induce innate immune activation.

C. acnes is one of the most abundant microbes in the skin from adolescence. Human epidermal keratinocytes recognize the presence of the 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. 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. 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. 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. Overall, these events contribute to the induction of the characteristic inflammatory symptoms during acne vulgaris pathogenesis in adolescents.

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. 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.

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 acts at different levels of the cascade. 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 single Ig and TIR domain containing - SIGIRR, Toll Interacting Protein - TOLLIP, TNF alpha induced protein 3 - TNFAIP3 and TNFAIP3 interacting protein 1 - TNIP1, acting at different levels of the TLR signaling cascade.

SIGIRR, TOLLIP, TNFAIP3 and TNIP1 are widely expressed in several cell types, among others, in cells that have direct contact with the human microbiota. Their role has been proposed in various pathogen infection models and the maintenance of tissue homeostasis.

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 selected, well-known negative regulators of the 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.

Materials and methods

- Human immortalized keratinocyte cell line HPV-KER, normal human keratinocytes (NHEK) and *ex vivo* organotypic skin (OS) cultures were used for our experiments.
- For bacterial treatment live *C. acnes* bacterium strains, belonging to different phylogenetic groups within the species (889, I/A, ATCC 11828, II, 6609, I/B) were used in a different multiplicity of infection (MOI).
- 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.
- In the identification of signaling pathways, playing a role in the regulation of the negative regulators, selective inhibitors of JNK (sp 600125), NF-κB (Bay 11-7085), p38 (sb 203580), ERK1/2 (PD 098059), STAT1 (Fludarabine) and STAT3 (Stattic) or, as a control, DMSO was applied to the cells for 1 hour.
- For the overexpression studies, cells were transfected using the X-tremeGENE 9 DNA Transfection Reagent with empty pcDNA3.1 vector, or pcDNA3.1-TNIP1 construct, into which TNIP1 cDNA sequences had been inserted.
- For transient siRNA-mediated gene silencing, ON-TARGETplus SMARTpool TNFAIP3-siRNA, TNIP1-siRNA, SIGIRR-siRNA, TOLLIP si-RNA or ON-TARGETplus Non-targeting Pool constructs were delivered by Santa Cruz siRNA Transfection Reagent
- NF-κB promoter activity was measured by luciferase reporter assay.
- Secreted cytokine and chemokine levels were measured by enzyme-linked immunosorbent assay (ELISA)
- Total RNA was isolated using TRI-Reagent and phenol-chloroform extraction. cDNA synthesis was performed, and changes in mRNA expression were detected by real-time RT-PCR.

- Protein expression changes were monitored by western blot analyzes and fluorescence microscopic analyzes.
- Tissue TNFAIP3 gene expression was analyzed using publicly available microarray data from the GEO Profile Database (GDS2478 datasets, ID: 33444972).
- Statistical analyzes: 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.

Results

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

First, we analyzed SIGIRR expression changes in human *in vitro* cultured keratinocytes co-cultured with the *C. acnes* 889 strain (MOI=100). We found that SIGIRR is expressed in HPV-KER cells, but its mRNA expression remained unchanged after C. *acnes t*reatment.

To determine whether strain-specific variation and dose-dependency occur, we applied *C. acnes* strains, belonging to various phylogenetic groups within the species (*C. acnes* 889, 6609, ATCC 11828, Group IA, IB and II.) in different MOIs (25, 100, 300) and compared the effects. None of the applied *C. acnes* strains induced SIGIRR mRNA expression changes at the used bacterial doses.

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

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. We found that neither basal nor the bacterium-induced TNFα levels were unaffected.

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.

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* 889, ATCC11828 and 6609 treatment using similar experimental setups. We found that TOLLIP mRNA levels remained unchanged. We observed similar results by analyzing TOLLIP protein levels upon *C. acnes* 889 treatment.

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.

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

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

To analyze possible strain-specific regulation of TNFAIP3, we co-cultured HPV-KER cells with the *C. acnes* 889, 6609 and ATCC 11828 strains, and compared their effects. We did not observe any strain-specific differences: TNFAIP3 mRNA expression levels were similar, independent of the strain used. Based on these observations, we used the *C. acnes* 889 strain in the subsequent experiments.

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

We also analyzed whether changes in *C. acnes*-induced TNFAIP3 expression, were dose-dependent, by co-culturing HPV-KER cells with the *C. acnes* 889 strain using different MOIs. We found that the extent of TNFAIP3 mRNA and protein expression changes depended on the bacterial dose.

To identify possible signaling pathways that are involved in *C. acnes*-induced TNFAIP3 regulation in keratinocytes, we investigated the contribution of selected pathways playing important roles in the bacterium-induced signaling events. We inhibited the NF-κB and MAPK (JNK, p38, and ERK1/2) signaling pathways 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 bacterium. We found that inhibition of the JNK signaling pathway decreased basal TNFAIP3 mRNA expression, whereas *C. acnes*-induced mRNA levels were affected by JNK and NF-κB inhibition. We also detected decreased protein levels upon JNK and NF-κB pathway inhibition in the *C. acnes*-treated samples.

To analyze the role of TNFAIP3 in the regulation of key inflammatory mediators, siRNA-mediated silencing was performed and downstream targets of TLR signaling pathways were monitored. We found that TNFAIP3 knockdown increased the basal NF-κB promoter activity and subsequently the basal and bacterium-induced mRNA expression of TNFα, IL-1α, IL-6, IL-8, CCL5 and the secreted protein levels of IL-6 and IL-8.

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. We observed elevated TNFAIP3 mRNA and protein expression levels in the epidermis part of OS cultures upon *C. acnes* treatment, similar to what we observed in monolayer cultures.

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. We found significantly increased levels of TNFAIP3 mRNA expression in the lesional skin samples compared to non-lesional skin of acne patients.

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

Next, we analyzed TNIP1 expression changes in HPV-KER cells upon *C. acnes* treatment. We found that TNIP1 is also 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.

Next, we applied different bacterium 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. 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.

We also 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. Elevated TNIP1 protein levels were also observed by immunofluorescence staining after 24 hours of *C. acnes* (MOI=100) treatment. Similarly to the mRNA levels, the abundance of TNIP1 protein increased in parallel with increasing *C. acnes* 889 doses.

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 JNK, NF-κB, p38, ERK1/2, STAT1, and STAT3 signaling pathways and performed *C. acnes* challenge. We found that basal 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.

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.

cDNA-based transient overexpression significantly decreased basal and *C. acnes*-induced NF-κB promoter activities, as a consequence, mRNA expression of basal and *C. acnes*-induced TNFα, IL-8 and CCL5 also decreased. Besides, bacterium-induced mRNA levels of IL-6 decreased, whereas IL-1α levels were not affected. Overexpression of TNIP1 also decreased IL-6, IL-8 and CCL5 protein secretion. In contrast, siRNA-mediated silencing of TNIP1 led to significantly increased constitutive and bacterial-induced NF-κB promoter activities. Basal and *C. acnes*-induced expression of TNFα, IL-8, and CCL5 mRNA markedly increased, whereas IL-1α and IL-6 expression increased moderately in response to TNIP1 silencing. Constitutive and bacterium-induced secretion of IL-8, IL-6, and CCL5 were also elevated in TNIP1 silenced HPV-KER cells.

The TNIP1 promoter contains retinoic acid response elements (RARE) and retinoid-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. 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 and significantly increased TNIP1 protein expression. In addition, basal and *C. acnes*-induced mRNA expression of TLR-2 and the pro-inflammatory TNFα and CCL5 decreased upon ATRA treatment. In contrast, both basal 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.

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 OS cultures and found similar results we observed in case of HPV-KER immortalized keratinocytes. TNIP1 mRNA and protein levels increased in response to *C. acnes* treatment in NHEK cells, whereas elevated protein levels were detected in the epidermal part of OS cultures.

We also applied ATRA to the upper, epidermal part of OS models. We found that TNIP1 protein expression levels increased in all epidermal layers 24 hours after drug treatment, in a manner similar to observed for immortalized keratinocytes.

Summary

- We found that even though SIGIRR and TOLLIP are expressed in HPV-KER cells, they do not seem to play important roles in the control of *C. acnes*-induced signaling cascades in keratinocytes. Their expression remained unchanged upon *C. acnes* challenge and the bacterium-induced inflammatory cytokine levels were not affected in SIGIRR or TOLLIP knockdown cells.
- TNFAIP3 and TNIP1 expression were rapidly induced in response to *C. acnes* in HPV-KER, NHEK cells and OS models.
- Expression changes of TNFAIP3 and TNIP1 were dependent on the *C. acnes* dose, but not on the used bacterium strain.
- Bacterium-induced changes in TNIP1 expression were regulated by signaling pathways involving JNK, NF-κB, p38 and ERK1/2, whereas TNFAIP3 expression was regulated by JNK and NF-κB pathways.
- Experimental modification of TNIP1 and TNFAIP3 levels affected the activity of the 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 entire epidermis.
- ATRA also reduced constitutive and bacterium-induced levels of TNFα, CCL5, and TLR2, while simultaneously increasing IL-8 and TLR4 expression.
- By analyzing publicly available microarray data in the GEO Profile database, we also showed that in acne lesions, TNFAIP3 mRNA expression was elevated compared to non-lesional skin samples from the same individuals.

Based on our findings, TNIP1 and TNFAIP3 play important roles in the regulation of TLR-induced signaling pathways in keratinocytes, and, through these roles, contributes to the control of *C. acnes*-induced innate immune and inflammatory events.

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