UNIVERSITY OF SZEGED, FACULTY OF MEDICINE
DEPARTMENT OF DERMATOLOGY AND ALLERGOLOGY
DOCTORAL SCHOOL OF CLINICAL MEDICINE

# The effect of *Cutibacterium acnes* on the barrier properties of *in vitro* cultured keratinocytes

Ph.D. Thesis

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## Publications related to the subjects of the thesis

I. **Bolla, B. Sz.;** Erdei, L.; Urbán, E.; Burián, K.; Kemény, L.; Szabó, K.: *Cutibacterium acnes* regulates the epidermal barrier properties of HPV-KER human immortalized keratinocyte cultures SCIENTIFIC REPORTS 10 : 1 Paper: 12815 , 13 p. (2020) **IF: 3.998**

*(Independent citation:0 Self citation:0 Cumulative: 0)*

*(Journal specialization: Scopus – Multidisciplinary, Location: Q1/D1)*

II. Szabó, K.; **Bolla, B. Sz.**; 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) **IF:0.000**

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## Other publications

III. L. Erdei, **B. Sz. Bolla, R. Bozó, G. Tax, E. Urbán, L. Kemény, K. Szabó:** TNIP1 Regulates *Cutibacterium acnes*-Induced Innate Immune Functions in Epidermal Keratinocytes. Front Immunol. 2018; 9: 2155. **IF: 4.534** *(Independent citation:3 Self citation:2 Cumulative: 5) (Journal specialization: Scopus – Immunology, Location: Q1)*

IV. **Manczinger, M.; Bodnar, V.; Papp, B. T.; Bolla, B. S.; Szabo, K.; Balazs, B.; Csanyi, E.; Szel, E.; Eros, G.; Kemeny, L.:** Drug repurposing by simulating flow through protein-protein interaction networks. CLINICAL PHARMACOLOGY & THERAPEUTICS 103 : 3 pp. 511-520. , 10 p. (2018) **IF: 6.336** *(Independent citation:1 Self citation:0 Cumulative: 1)*

*(Journal specialization: Scopus – Pharmacology, Location: Q1)*

V. **Megyeri, K; Orosz, L; Bolla, S; Erdei, L; Razga, Z; Seprenyi, G; Urban, E; Szabo, K; Kemeny, L.:** *Propionibacterium acnes* induces autophagy in keratinocytes: involvement of multiple mechanisms JOURNAL OF INVESTIGATIVE DERMATOLOGY 138:4 pp. 750-759., 10 p. (2018) **IF: 6.290** *(Independent citation:5 Self citation:2 Cumulative: 7)*

*(Journal specialization: Scopus – Dermatology, Location: Q1)*

VI. **Szabo, K ; Erdei, L ; Bolla, BS ; Tax, G ; Biro, T ; Kemeny, L.:** Factors shaping the composition of the cutaneous microbiota BRITISH JOURNAL OF DERMATOLOGY 176:2 pp. 344-351., 8 p. (2017) **IF: 6.129** *(Independent citation:16 Self citation:5 Cumulative: 21)*

*(Journal specialization: Scopus – Dermatology, Location: Q1)*

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**Abbreviations**

| | |
|---|---|
| **AB/AM** | antibiotic/antimycotic |
| **BP** | benzoyl peroxide |
| ***C. acnes*** | *Cutibacterium acnes* |
| **CAMP** | Christie–Atkins–Munch-Peterson factor |
| **Ci/nCi** | Cell index/ normalized cell index |
| **CLDN 1, 4, 7** | claudin 1, claudin 4, claudin 7 |
| **CXCL8/IL-8** | interleukin 8 |
| **HPV-KER** | HPV immortalized human keratinocytes |
| **HRP-conjugated** | horseradish peroxidase -conjugated |
| **IgG** | immunoglobulin G |
| **IHC** | immunohistochemical staining |
| **KSFM/KC-SFM** | keratinocyte serum free medium |
| **LY** | Lucifer yellow |
| **NHEK** | normal human epidermal keratinocyte |
| **OCLN** | occludin |
| **OS model** | organotypic skin model |
| **PA** | propionic acid |
| **PBS** | phosphate-buffered saline |
| **PCR** | polymerase chain reaction |
| **PGN** | peptidoglycan |
| **PRR** | pathogen recognition receptor |
| **PSU** | pilosebaceous unit |
| **RNA/mRNA** | ribonucleic acid/ messenger ribonucleic acid |
| **RT** | room temperature |
| **RT-PCR** | reverse transcription polymerase chain reaction |
| **RTCA system** | real time cell analysis system |
| **SCFA** | short-chain free fatty acid |
| **SDS-PAGE** | sodium dodecyl sulfate- polyacrylamide gel |

| | |
|---|---|
| | electrophoresis |
| **SEM** | standard error of the mean |
| **TEER** | transepithelial electrical resistance |
| **TJ** | tight junction |
| **TLR2** | Toll-like receptor 2 |
| **TLR4** | Toll-like receptor 4 |
| **TNFα** | Tumor necrosis factor alpha |
| **ZO-1, 2, 3** | zonula occludens-1, 2, 3 |

# 1. Introduction

## 1.1. The skin

The human skin not only separates the inner part of the body from the external environment but also provides an opportunity to sense the changes in the outer world [1–4]. It has a role in thermoregulation, and based on the anatomical structure, provides a complex, **physical or mechanical barrier** to prevent mechanical and chemical injuries of the body. Together with the cells of the immune system, it also plays important roles in the formation of an **immunological barrier**, offering protection against microbial invasion and helping the restoration of an intact tissue after wounding. It is in direct contact with the environment, and acquires a specialized microbial community, called **microbiota**. These microbes help in preventing the colonization of pathogenic microbes, thus with the skin, form a **microbial barrier**. This community may also aid the differentiation and maturation of a functionally full-fledged tissue and play important roles in the maintenance of skin homeostasis [5–7].

## 1.2. The anatomical structure of the human skin

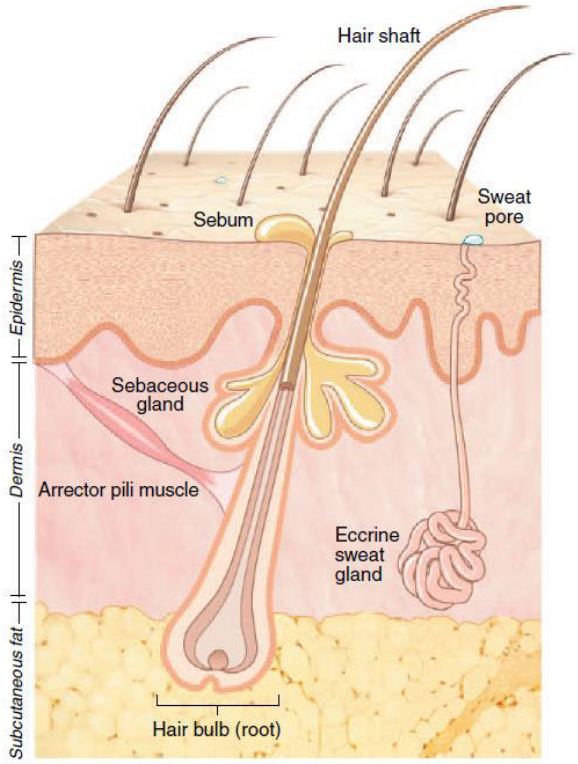


**Figure 1. The anatomical structure of the skin**

The human skin is composed of three different layers: the epidermis, dermis, and the hypodermis, each having various functions in our body (Fig. 1.). The outermost layer of the skin is the epidermis, which is built up by keratinocytes. It has different layers, depending on the differentiation state of the cells. The first layer is located above the basement membrane, called *stratum basale*. It contains biologically active, proliferating cells, which continuously divide to provide new cells to the upper layers and help the rejuvenation and regeneration of the tissue. The next layer is the *stratum spinosum*, where the

keratinocytes start to differentiate. Above this, one finds the *stratum granulosum*, where the cells further differentiate, leading to marked cell shape changes [8]. The last layer of the epidermis is the *stratum corneum*. This structure consists of dead, cornified cells, which are continuously shedding upon mechanical contact. It provides important physical and mechanical protection, and cell detachment also helps to regulate the colonization of various microbes by removing them from the surface [9].

Under the epidermis lays the dermis. This tissue is composed of fibroblasts, containing two regionally distinct areas, the superficial papillary and the deeper reticular dermis. While the upper part closely interacts with the epidermis, the reticular dermis is in connection with the deeper skin layers. It has important functions not only in providing nutrients to the epidermis but also containing the different skin appendages, including the hair follicles, eccrine, sweat, and apocrine glands [9]. The innermost part is the hypodermis, which consists of loose connective and adipose tissues, together with different cell types, such as fibroblasts, adipocytes, and macrophages. This region not only protects from mechanical stress but also plays a role in thermoregulation, mainly during cold exposure and exercise [10].

### 1.3. The structure and properties of the epidermal barrier and the tight junctions

In the epidermis, the keratinocytes are tightly packed to one another in the different layers, even though their morphological appearances continuously change according to their

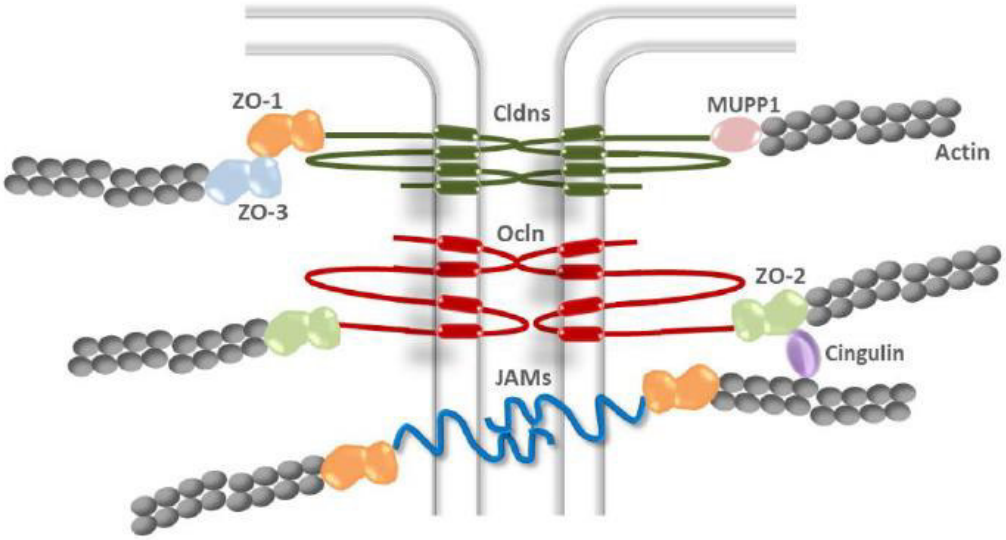


**Figure 2. The localization of the tight junction proteins in the stratum granulosum.** (Bäsler *et al.,* 2016 [14])

differentiation state. The cells are also connected by specialized cell junctions, which further

help to seal the space and aid the establishment of skin barrier functions [1]. These specialized cell junctions are the hemidesmosomes, desmosomes, gap junctions, adherent junctions, and tight junctions (TJ)[1,11–14]. The hemidesmosomes are mostly located in the basal layer of the epidermis and provide a connection to the dermis. Desmosomes, gap- and adherens junctions are present in all epidermal layers. While all of them connect keratinocytes, gap junctions have an additional role in cellular communication, while adherens junctions further seal the space between the cells and provide the necessary conditions for TJ formation [13].

TJs are multiprotein junctional complexes that are formed between neighboring cells and seal the space (paracellular space) between them, and control the transport through the tissue (Fig. 2) [15]. These complexes are located only in the 2nd layer of the *stratum granulosum* as functional sealing complexes, forming intercellular contact sites, so-called 'kissing points' between the cells (Fig. 3).

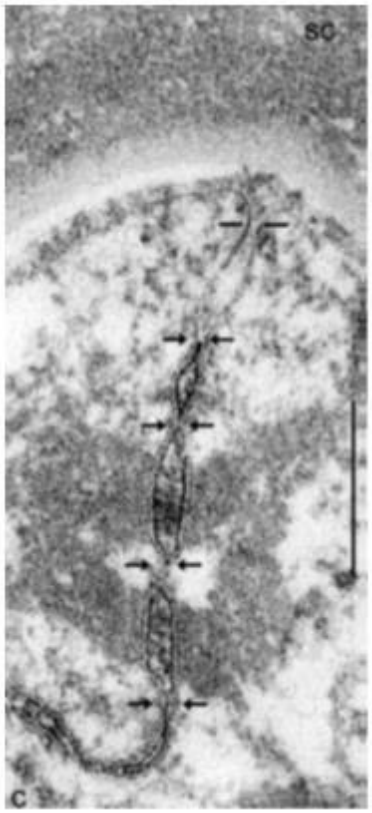


**Figure 3. Electron microscopic image of TJ complexes (arrows) in ultrathin sections of the fetal plantar epidermis** (J. Brandner, 2002 [12])

TJs are consist of at least 40 different proteins. A majority of them are either transmembrane or adaptor molecules[16,17]. The most important structural components belong to the claudin (CLDN) protein family. These are four-transmembrane proteins, which form the structural parts of the TJs, called strands. These provide selectivity for the complexes and determine which ions and molecules can pass through the central pore [18]. Occludin (OCLN) also belongs to the transmembrane molecules, and similarly to the CLDN protein family, has four transmembrane domains. It forms indirect interactions with the actin cytoskeleton and directly binds several intracellular proteins [19]. The junctional adhesion molecules (JAMs) also serve as transmembrane molecules, but their structure is different from the CLDN and OCLN family. They have only one transmembrane domain and interact with adaptor molecules not only in the TJ but also in adherent junctions. The most extensively studied and characterized adaptor molecules are zonula occludens 1, 2, and 3 (ZO-1, 2, and 3), cingulin, and afadin. These molecules serve as a link between the TJ complex and the cytoskeleton and also to signaling cascades to maintain a dynamic cell integrity [15].

In the epithelial tissues, TJ pairs form a zip lock between adjacent cells. This structure generates a tight barrier, which prevents the free transportation of molecules and ions through the paracellular space. Their regulated movement is called the paracellular transport pathway[20]. Two major forms of these transport mechanisms are known. The pore pathway is highly conductive, allows the movement of ions and small charged molecules through diffusion, electrodiffusion or osmosis, because of the gradients that exist between the two sides of the membrane [21]. TJs can regulate the properties of this transport pathway through their protein composition, for example, through the CLDN content of the TJ strands [22].

Large molecules and macromolecules can also pass through the TJ barrier under certain circumstances through the leak pathway. It is a charge non-selective transport method, a low-capacity route, which regulates the passage of larger molecules. This pathway depends on the presence of transient breaks within the TJ fibril network and is often measured by the passage of fluorescent tracers across epithelial monolayers [22,23].

These transport pathways play important roles in the regulation of barrier functions but also contribute to the determination of apical and basolateral polarization, in signaling events through the different adapter molecules, and also affect cellular behavior and differentiation processes [24].

### 1.4. The cutaneous microbiota, composition, localization, and function

The cutaneous microbiota is composed of diverse bacterial and fungal species, viruses, and mites. This community is located mostly on the skin surface, but also colonize the hair follicles [25]. In the different niches, which are provided by the skin, the most dominant microbes are belonging to the bacterial kingdom. Among these, the most abundant phyla are the *Actinobacteria* (51.8%), *Firmicutes* (24,4%), *Proteobacteria* (16,5%), and *Bacteroides* (6,3%). On the genus level, the majority of the identified species belong to the *Corynebacterium*, *Cutibacterium*, and *Staphylococcus* genera [26].

The exact composition of the microbial communities is strongly dependent on the niche where they reside, also on the body site, age, and environmental influence [27]. Oily skin regions, where the number of sebaceous glands is high (face, upper chest, and back), are predominantly inhabited by *Corynebacterium*, *Cutibacterium*, and *Staphylococcus* species. In moist sites, we most frequently find *Corynebacterium*, *Staphylococcus*, and *Betaproteobacteria* species. In contrast to that, dry areas have mixed populations of B*etaproteobacteria* and *Flavobacteriales* [28,29].

The cutaneous microbiota is in constant contact with the skin cells and provides factors to modulate the skin milieu. By adapting to the different cutaneous niches, they protect us from potentially harmful pathogens by competing for nutrients, habitats, and by limiting their growth. They use the lipids secreted by the keratinocytes and sebocytes to gain energy. Through their metabolism, they produce free-fatty acids to modulate the surrounding milieu. The produced free-fatty acids help to maintain the acidic pH of the skin, which further limits the colonization of the skin with pathogen microbes. These microbes come into contact not only with keratinocytes but also with cells from the immune system. Through these connections, they play important roles in the establishment and maturation of cutaneous immune functions. [29–32].

### 1.5. *Cutibacterium acnes* (*C. acnes*) is a prominent member of the cutaneous microbiota in healthy skin and acne pathogenesis

*Cutibacterium acnes* (*C. acnes;* formerly known as *Propionibacterium acnes*) is a rod-shaped, Gram-positive, anaerobic, but aero tolerant bacterium [33]. According to our current knowledge, we can determine six different phylotypes within the species, named as type IA1, IA2, IB, IC, II, and III [34]. This categorization is based on the genomic sequences of the different strains and also their biological activity.

*C. acnes* preferentially resides in oily skin regions, as it uses different sebum components for its growth. Bacterial colonization starts right after birth, and the bacterium becomes a dominant species in puberty when sebaceous gland activity increases due to the hormonal changes [26].

In healthy skin, *C. acnes* is a member of the healthy cutaneous microbiota. During its metabolism, the bacterium produces short-chain free fatty acids (SCFAs). These molecules play immunoregulatory functions, help to maintain the acidic pH in the skin, and inhibit the growth of potential pathogens like *Staphylococcus aureus* [35]. *C. acnes* strains also produce a bacteriocin-like substance called acnecin, which acts against not only other *C. acnes* strains but also other species and restricts their growth [36].

The bacterium also affects keratinocyte functions. It can stimulate their proliferation in monolayer cultures, and by affecting the expression of genes with a role in the keratinocyte differentiation processes, it may also contribute to the maturation of the fully functional epidermal tissue [5,6]. *C. acnes* also induces innate immune and inflammatory events in

keratinocytes through the activation of Toll-like receptor 2 and 4 (TLR2, TLR4). Extensive activation of these pathways leads to the pathogenesis of *acne vulgaris* [37,38].

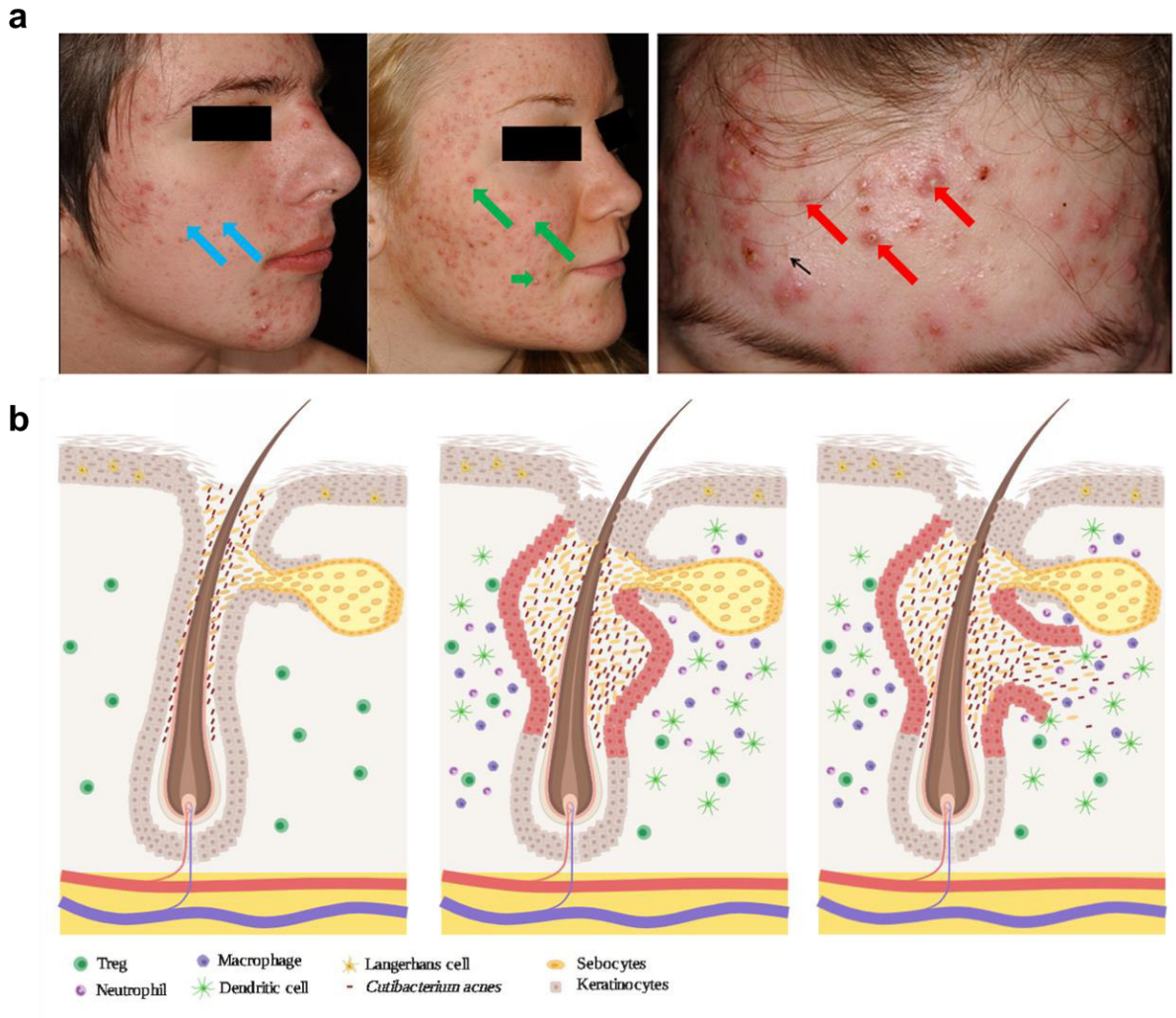


**Figure 4. Clinical characteristics (a) and schematic representation (b) of *acne vulgaris*.** Depending on the severity of *acne vulgaris,* the clinical features differ (a). In patients with mild acne, comedos (blue, black arrows) are present in the affected area. In moderate acne patients, papules and pustules (green arrows) are dominating, while in severe acne, nodules and cysts (red arrows) also appear. (Photos are part of the collection of the Department of Dermatology and Allergology, University of Szeged. Schematic figure was created using the Biorender online software tool, http://www.BioRender.com)

Acne is the most common, multifactorial, inflammatory skin disease, which affects a large proportion (approximately 85%) of the adolescent population. Its pathogenesis is complex (Fig. 4). It starts parallel with the puberty induced hormonal changes and elevated androgen hormone levels, which leads to increased sebum secretion and abnormal *C. acnes* colonization of the pilosebaceous units (PSUs) or follicles. According to the current view, initial steps in

acne pathogenesis include keratinocytes hyperproliferation and abnormal differentiation, which increases the amount of terminally differentiated, dead cells in the follicle. As the growing amount of cell debris cannot be emptied to the skin surface fast enough, together with the continuously generated sebum, it will form a plug. This mechanical obstruction will stall further outflow from the follicle, leading to the formations of comedos in the skin. These events gradually increase anaerobic conditions in the PSU, providing ideal conditions for further *C. acnes* growth. Enhanced induction of innate immune and inflammatory reactions within the PSU and gradually in the surrounding tissues will finally lead to the establishment of papules and pustules. The retained sebum, together with the growing number of bacterial cells exerts a gradually increasing pressure to the inflamed follicle wall, finally causing the rupture of it. Based on this model, bacterial dysbiosis, the abnormal interaction of skin cells, among others, keratinocytes, sebocytes, and the *C. acnes* bacterium is crucial in acne pathogenesis. Bacterium-induced immune activation in keratinocytes is a key step in lesion formation. Currently, however, is not known whether and how the epidermal barrier is affected in these processes.

## 2. Aims

In our studies, we aimed to investigate the effect of *C. acnes*, which is a prominent member of the cutaneous microbiota, on keratinocyte barriers. We specifically analyzed the following questions:

- How does *C. acnes* affect the barrier properties of confluent *in vitro* keratinocyte monolayer cultures, representing various differentiation states?
- What are the underlying cellular and molecular changes?
- Does *C. acnes* has any effect on the barrier properties of *ex vivo* cultured organotypic skin models?

## 3. Materials and methods

### 3.1. *C. acnes* strains and culture conditions

*C. acnes* 889 (type IA), 6609 (type IB), and ATCC11828 (type II) strains were cultured and stored as previously described in detail [32,35].

### 3.2. Keratinocyte cell cultures, organotypic skin models and *C. acnes* treatment

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

The human immortalized keratinocyte cell line HPV-KER [35] and NHEKs were used for our experiments. Both cell types 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 $CO_2$). Differentiation of confluent NHEK and HPV-KER cultures was induced with the addition of 1.7 mM $CaCl_2$.

For generating the organotypic skin (OS) models, 1 $cm^2$ whole-thickness skin samples were placed onto the nitrocellulose membrane of a 6-well insert, with the dermal surface placed on the membrane. Samples were cultured in air-liquid interphase using Dulbecco's Modified Eagle's/F12 medium supplemented with 10% fetal bovine serum. *C. acnes* culture (15 µl, $10^9$ CFU/sample) was transferred to skin samples (epidermal surface) and further cultured for an additional 24, 48, or 72 hours. Samples for immunofluorescent staining were fixed in 4% paraformaldehyde and embedded in paraffin. For PCR analysis, 6 mm punch biopsies were taken from the samples and the epidermis was separated from the dermis by incubating in dispase solution for 3 hours at 37°C. Epidermis samples were homogenized using the Ultra Turrax T8 homogenizer (IKA-WERKE). Total RNA was isolated using TRI-Reagent (Molecular Research Center; Cincinnati, USA).

### 3. 3. Real-time, label-free cellular analysis of keratinocyte cultures

The integrity and the barrier properties of HPV-KER and NHEK monolayer cultures were detected in real-time using an impedance-measurement based technology (xCELLigence, Real Time Cell Analyser system, ACEA Biosciences, San Diego, USA)31,56. For contact inhibited cultures, NHEK and HPV-KER cultures were grown to confluency on fibronectin-coated 96-well E-plates (Ca-low cultures). After 24 hours of growth, cells were co-cultured with live *C. acnes* strains belonging to different phylogenetic groups within the species (889, ATCC11828, 6609) at various multiplicity of infection (MOI). Each treatment was performed in five technical replicates. Impedance (Z) values were measured every 60 minutes (unless otherwise noted), from which a dimension-free cell index (Ci) was calculated (Ci = [impedance at time point n – background impedance without cells]/ nominal impedance value). In the used confluent cultures, Ci values depend on the applied cell number, the cell-cell adhesion and the cell-surface interaction. In some experiments, normalized Ci (nCi) values were also determined (nCi = Ci values at time point n / Ci values at a selected time point, e.g., time point of applied treatment). Ci and nCi were plotted as a function of time. Each data point represents the mean ± the standard error of the mean (SEM).

For the differentiated NHEK and HPV-KER (Ca-high) cultures, KC-SFM culturing media was supplemented with 1.7 mM $CaCl_2$ after the cultures reached confluency. Forty-eight hours after treatment, the differentiated cultures were treated with *C. acnes* strains, and barrier changes were monitored as described above.

The effect of propionic acid (PA) was determined using HPV-Ker (Ca-low) cultures. After a confluent state was reached, PA was added to the cells at different concentrations (1, 5, 7.5, 10mM), and barrier changes were monitored as described above.

Each treatment was performed in five technical replicates, and the reported data points represent the mean ± SEM unless otherwise noted.

### 3. 4. Western blot analysis of TJ proteins

Cells were scraped with a cell scraper and harvested by centrifugation. The pellets were resuspended in protein lysis buffer (20 mM HEPES, 150 mM $KCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol, 5% TritonX-100, 10% glycerol, 0.1% NP-40) containing 1% protein inhibitor cocktail, 1% phenylmethylsulfonyl fluoride, 0.5% sodium dodecyl sulfate (all components from Sigma). Protein concentrations were measured with the BCA detection kit (Thermo Scientific, Waltham, MA, USA). SDS-PAGE was carried out using 50 µg protein sample, and proteins

were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with Tris-buffered saline solution (150 mM NaCl, 25 mM Tris, pH 7.4) containing 3% and 5% non-fat dry milk powder (Bio-Rad). Rabbit anti-human actin (Sigma-Aldrich) and anti-ZO-1 (Thermo Fisher Scientific) antibodies were diluted at 1:1000, mouse anti-claudin 1 (Abnova), anti- occludin (Abnova), and anti-Claudin-4 (Thermo Fisher Scientific) were diluted at 1:500, and membranes were incubated with antibodies overnight at 4 °C. Anti-rabbit and anti-mouse IgG horseradish peroxides-conjugated (HRP-conjugated) secondary antibodies (SouthernBiotech) were applied, and the resulting bands were visualized using the C-DiGit Blot Scanner (LI-COR Biosciences, Nebraska, USA) or the Omega Lum G Chemidoc Imageing System (Aplegen, Inc, Pleasanton, CA, USA).

### 3. 5. Total RNA isolation and real-time RT-PCR

Total RNA was isolated from the monolayer cultures using TRI-Reagent (Molecular Research Center; Cincinnati, USA). cDNA synthesis was performed using EvoScript cDNA Synthesis Kit (Roche), and changes in the expression of TJ genes (CLDN1, CLDN4, OCLN, and ZO-1) were analyzed by real-time RT-PCR using the Universal Probe Library system (Roche, Indiana, USA). Supplementary Table 1 lists the PCR protocols and primer sequences used. TaqMan Gene Expression Assays (Thermo Fischer Scientific, Massachusetts, USA) were used to detect tumor necrosis factor α (TNFα; HS00174128_m1) and CXC motif chemokine ligand 8 (CXC8; HS00174103_m1). All data were normalized to the 18S rRNA using the $\Delta\Delta C_t$ method before comparing to the time-matched untreated control samples. Table 2. lists the used primers and probes.

| **Applera** | **Temperature** | **Time** | **Cycle** |
|---|---|---|---|
| **1** | 95 °C | 6 min | x1 |
| **2** | 95 °C<br>60 °C | 15 s<br>1 min | x40 |
| **UPL** | | | |
| **1** | 95 °C | 3 min | x1 |
| **2** | 95 °C<br>58 °C | 15 s<br>1 min | x40<br>(18 S rRNA x25) |
| **3** | 37 °C | 10 min | x1 |

**Table. 1 Used PCR protocols**

| Gene | Sequence | Probe number |
|---|---|---|
| 18 S rRNA | F:5'CGCTCCACCAACTAAGAACG3'<br>R:5'CTCAACACGGGAAACCTCAC3' | 77 |
| Claudin 1 (CLDN1) | F: TTGACTCCTTGCTGAATCTGAG<br>R: GGCCACAAAGATTGCTATCAC | 79 |
| Claudin 4 (CLDN4) | F: TCACACCTGGGTCCCCTA<br>R: TCGCTTTAACCTGGGAGATG | 19 |
| Occludin (OCLN) | F: GTCATCCAGGCCTCTTGAAA<br>R: GGTGCATAATGATTCGGTTTG | 10 |
| Zonula occludens 1 (ZO-1) | F: TCAGACAGGCGGTCAGTG<br>R: ATATGGCTTGCCAATCGAAG | 20 |

**Table 2. List of the used primers and probes.**

### 3. 6. Immunohistochemical staining

Paraffin-embedded OS samples were used to analyze changes in TJ protein levels after *C. acnes* 889 treatment. A Bond-Max automated IHC/ISH stainer (Leica Biosystems, Wetzlar, Germany) was used for immunohistochemical (IHC) staining. The staining protocol is described here briefly. Six μm sections were cut, placed on glass slides, deparaffinized (dewax, 72°C), and rehydrated. Subsequently, antigen retrieval was performed (10mM citrate buffer, pH 6.0, 100°C, 20 min). Primary antibodies against TJ proteins [CLDN1 (Abcam, Cambridge, UK), CLDN4 (Thermo Fischer Scientific, Massachusetts, USA), OCLN (Abnova, Taiwan), ZO-1 (Thermo Fischer Scientific, Massachusetts, USA)] were added to the samples at 1:100 dilutions (RT, 30 min), and also matching isotype controls [mouse IgG2a (Biolegend, CA, USA), rabbit poly IgG (Santa Cruz Biotechnology Inc, CA, USA) and mouse IgG1 κ (Biolegend, CA, USA)] which dilution rations were matched to the used primary antibodies concentrations. After primary antibody labeling, post-primary steps were performed for 8 min at room temperature (RT), subsequently, the polymer step was performed (15 min, RT). Peroxidase blocking was carried out (3 min, RT). Washing steps were performed before each step. Samples were developed with DAB-Chromogen for 10 min and dyed with hematoxylin. After covering with coverslips, samples were analyzed with a microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with an AxioCam MRm camera.

### 3. 7. Transepithelial electrical resistance measurement

Confluent HPV-KER monolayer cultures were grown on 12 mm transwell inserts (pore size 0.4μm, Corning, New York, USA) in KSFM media. Confluent Ca-low cultures were treated with *C. acnes* 889 strain (MOI=300) and transepithelial electrical resistance (TEER) values were measured in Hank's salt balanced solution (HBSS) using an Epithelial Volt/Ohm (TEER) Meter EVOM2 (World Precision Instruments, Sarasota, FL, USA) at 24 and 72 hours after treatment.

For the Ca-high cultures, confluent, contact-inhibited keratinocyte cultures were further differentiated by the addition of $Ca^{2+}$ (1,7 mM) to the KSFM culturing media and incubation for 72 hours. TEER values were measured at 24 and 72 hours after treatment with *C. acnes* 889 in HBSS buffer using an Epithelial Volt/Ohm (TEER) Meter EVOM2 (Sarasota, FL, USA).

### 3. 8. Trypan blue exclusion assay

HPV-KER cells were seeded on 12-well plates at a starting density of 300,000 cells/well. After 48 hours, the cultures were co-cultured with various doses of *C. acnes* 889 strain (MOI=100, 300). Samples were trypsinized and collected at 0, 24, 48 and 72 hours after treatment, washed with phosphate-buffered saline (PBS) and stained with trypan blue dye (Sigma-Aldrich, St. Louis, Missouri, United States). Viable cells were counted using a hemocytometer.

### 3. 9. Lucifer yellow penetration assay

HPV-KER cells were seeded on porous 12-well plates (pore size 0.4μm, Corning, New York, USA) at a density of 1.5 x $10^5$ cells/well using KSFM (Life Technologies, Carlsbad, USA). Ca-high cultures were established and treated with *C. acnes* 889 strain (MOI=300) for 24 and 72 hours. Lucifer yellow (LY) penetration experiments were carried out in HBSS containing 100 μM LY (Sigma-Aldrich, Saint Louis, Missouri, USA). HBSS-LY media was added to the upper chamber, and samples were collected from the bottom chamber after 30 min of incubation using standard culturing conditions (5% v/v $CO_2$, 37°C). Fluorescence intensities were measured with a BMG FLUOstar OPTIMA Fluorescence plate reader (Gemini BV Laboratory, Netherland) and relative fluorescence intensities were calculated. Fluorescence intensities were normalized to the time-matched untreated control values.

In the case of the OS models, *C. acnes* 889 strain (15 μl, $10^9$ CFU/sample) was pipetted onto the upper, epidermal part of the skin samples, which were then incubated for 72 hours. For

the transport experiments, LY diluted to 1 mM LY with PBS (15 µl/sample) was applied to the top. After 3 hours, skin samples were collected using 6 mm punch biopsies and embedded in Shandon Cryomatrix (Thermo Fischer Scientific, Massachusetts, USA) for frozen sectioning. Sections (6 µm) were cut from the samples, and LY dye penetration was analyzed using a Zeiss Axio Imager Z1 fluorescence microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with an AxioCam MRm camera.

### 3. 10. Antibiotic treatment of *C. acnes* co-cultured HPV-KER monolayers

The effect of antibiotic treatment of *C. acnes* co-cultured HPV-KER monolayers was observed in real-time using the RTCA system. HPV-KER cells were plated at a density of 30,000 cells/well in fibronectin-coated 96-well E-plates. After the cultures reached confluency, they were treated with *C. acnes* 889 strain (MOI: 100), and 44 hours later, 1% or 5% AB/AM was added to the cultures. Each treatment was performed in five technical replicates. Impedance values were measured every 60 minutes for 120 hours, and a dimension-free cell index (Ci) was calculated from the data. Ci (average of the technical replicates) tracings were normalized to values recorded at the addition of the bacterium to the cultures, and the resulting nCi values were plotted.

### 3. 11. Statistical analysis

Unless otherwise noted, all the data are presented as mean ±SEM for three independent experiments. For xCELLigence analysis, treatment was performed in at least five times. For real-time RT-PCR and trypan blue exclusion experiments, each treatment was performed at least three times. For western blot analysis and IHC staining, each condition was repeated once in each independent experiment. Data were compared using paired Student's t-test and one-way ANOVA with post-hoc Tukey-test. A probability value of less than 0.05 was considered significant.

## 4. Results

### 4. 1. $Ca^{2+}$-induced differentiation leads to marked nCi and corresponding barrier changes of *in vitro* keratinocyte cultures

Ohmic resistance and impedance measurements across a wide spectrum of frequencies are considered as good indicators of the integrity of cellular barriers [39–42]. Thus, we investigated how different culturing conditions affect the measurable impedance (Z), and the calculated Cell index (Ci) values of confluent NHEK (Fig. 5 a) and HPV-KER (Fig. 5 b) cultures using real-time cellular analysis and interpreted the changes as alterations in barrier properties of the *in vitro* monolayer cultures [40–42]. We allowed the cells to form confluent monolayers using basal KSFM and then raised the concentration of extracellular $Ca^{2+}$, which is known to induce keratinocyte differentiation [43]. We found that, after an initial growth phase, Ci values reached a plateau as the cultures became confluent and contact-inhibited. Replacing the media with fresh media containing high $Ca^{2+}$ concentrations (Ca-high) induced a marked and immediate increase in Ci compared to samples that were only contact inhibited and maintained in low $Ca^{2+}$ media (Ca-low). NHEK and HPV-KER cultures behaved similarly, suggesting that the latter cells may be used as a model to analyze keratinocyte barrier functions in monolayer cultures (Fig. 5 a and b). These findings also agree with the available literature data and suggest that high extracellular $Ca^{2+}$ concentration leads to the stabilization of keratinocyte barriers. In addition, the capability of the xCELLigence RTCA system for real-time monitoring of these properties was confirmed [44,45].

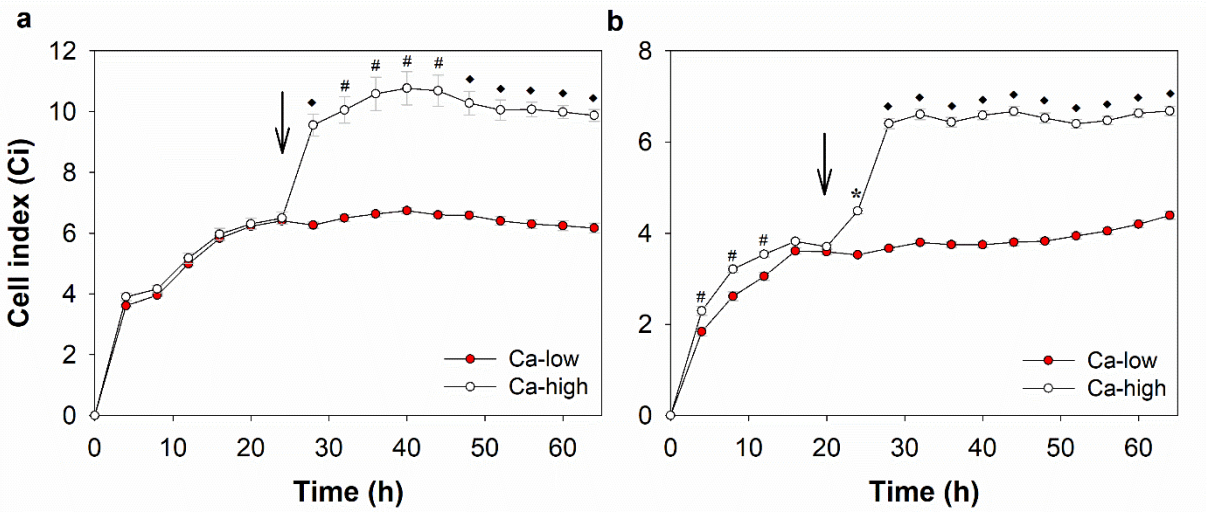


**Figure 5. High $Ca^{2+}$ concentration leads to elevated Ci values of NHEK and HPV-KER cultures.** Confluent NHEK (a) and HPV-KER (b) monolayer cultures were incubated in

standard KSFM media. After 24 hours (marked with an arrow), the extracellular $Ca^{2+}$ concentration was raised, leading to marked Ci increases. Representative image of three independent experiments. Data points are measured every 4 hours. Time points are the mean ±SEM. Statistical analysis with Student's t-test, *p< 0.05, #p< 0.005, ♦p< 0.0005.

### 4. 2. The effect of *C. acnes* on the integrity of *in vitro* keratinocyte cultures

We used the same experimental setup to analyze how the addition of *C. acnes* to the Ca-low and Ca-high NHEK and HPV-KER cultures affected keratinocyte barriers. For this, *C. acnes* 889 strain (MOI=100, 300) was added, and nCi values were recorded in every 4 hours for 95 hours (Fig 6).

In Ca-low NHEK (Fig. 6 a) and HPV-KER (Fig. 6 c) cultures, we first observed a rapid and transient increase of nCi values, suggesting improved barrier properties of the cultures. The peak in nCi was followed by a drop in the Ca-low cultures, suggesting that after reaching a threshold, continuously growing bacterial cells may have deleterious effects on the barrier properties of keratinocyte monolayers. We also repeated the experiments on differentiated, Ca-high NHEK, and HPV-KER monolayers. In these cell cultures, only the dose-dependent decrease of nCi values was detected, independent of the used cell type and *C. acnes* MOI (Fig. 6 b, d).

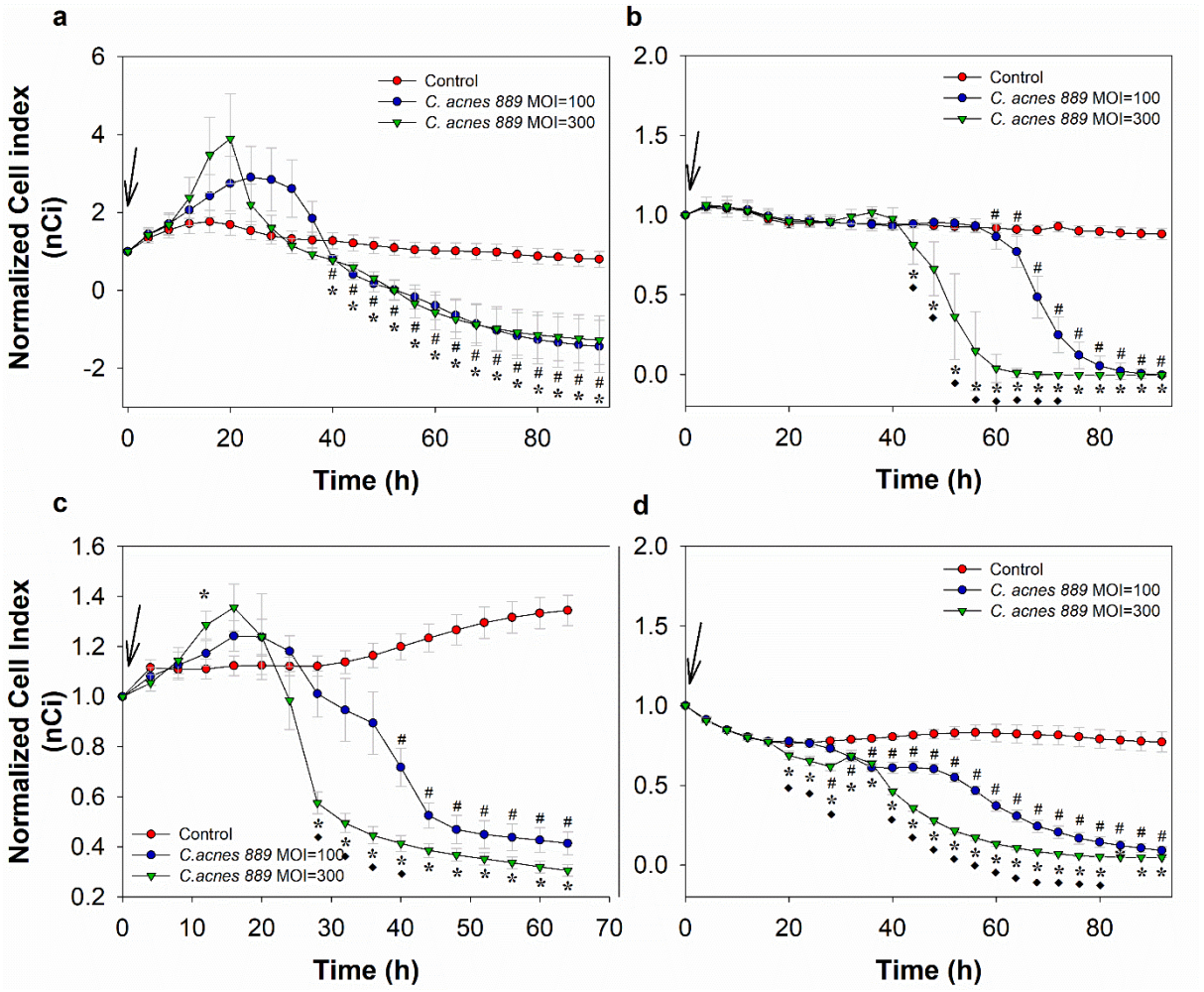


**Figure 6. Co-culturing HPV-KER and NHEK keratinocyte monolayers with *C. acnes* 889 strain leads to dose-dependent nCi changes.** Ca-low and Ca-high NHEK (a, b, respectively) and HPV-KER (c, d, respectively) keratinocyte monolayers were established and co-cultured with *C. acnes* 889 strain (0 hour time point, marked with an arrow) in different doses (MOI= 100, 300). Addition of the bacterium induced dose-dependent nCi changes in all cultures. (For detailed description, see the corresponding text.) Data points are measured in every 4 hours, representing the mean ± SEM. Statistical analysis with one-way ANOVA, post-hoc Tukey test, $p<0.05$, alpha=0.05, # control *vs. C. acnes* 889, MOI=100, *control *vs. C. acnes* 889, MOI=300, ♦ *C. acnes* 889, MOI=100 *vs. C. acnes* 889, MOI=300.

### 4.3. Cell number measurements of HPV-KER monolayers upon co-culturing with *C. acnes 889* strain

It is known from the literature that apart from the barrier properties, Ci changes may reflect differences in the number or the specific dimensions of cells attached to the electrodes. To determine the exact nature of the *C. acnes*-induced cellular events, we monitored the effect of the bacterium on the number of cells both in the Ca-low and Ca-high HPV-KER cell cultures.

No significant change in cell number was detected using a trypan blue exclusion assay (Fig. 7 a, b).

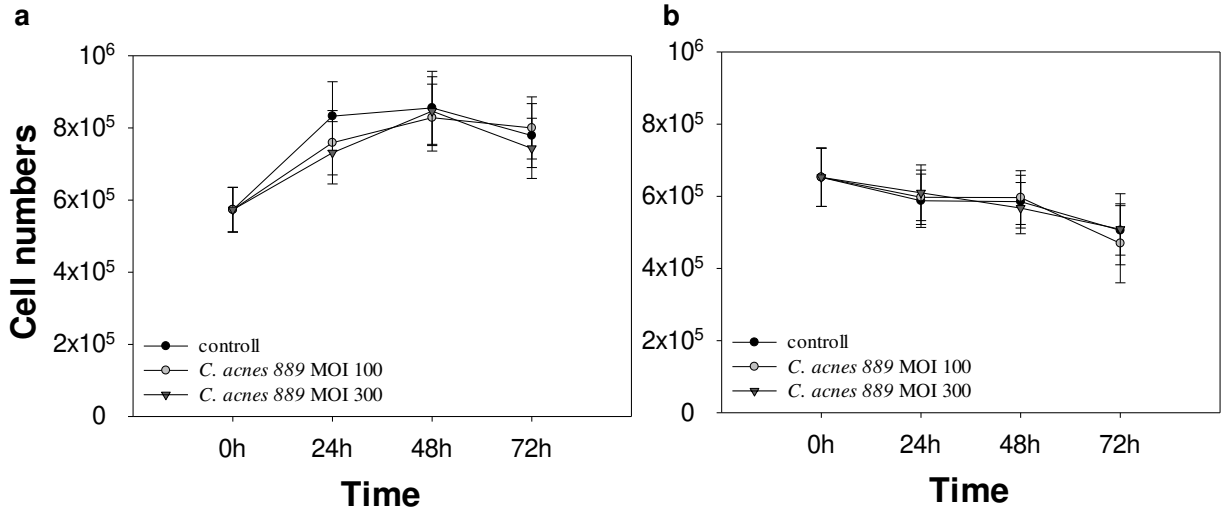


**Figure 7. Cell numbers do not markedly change in Ca-low (a) and Ca-high (b) HPV-KER monolayers upon co-culturing with *C. acnes* 889 strain.** No major changes were detected in the cell number in the different cultures after bacterial treatment, independent of the applied *C. acnes* doses. (Each treatment was performed in three technical replicates, and data points represent the mean ± SEM.)

### 4.4. Barrier integrity measurements of HPV-KER monolayers upon co-culturing with *C. acnes* 889 strain

We also performed TEER measurement, a widely accepted quantitative technique to analyze tight junction dynamics [39]. Selected time-points (24 and 72 hours) were chosen, based on our real-time investigation, and TEER measurement was performed using untreated samples as control and samples treated with *C. acnes* (MOI=300) in both Ca-low and Ca-high HPV-KER monolayers. Our results indicate that the TEER resistance values first increased in the Ca-low cultures, followed by a decrease in both models in the presence of the bacterium, similarly to what we found in the xCELLigence analysis (Fig. 8).

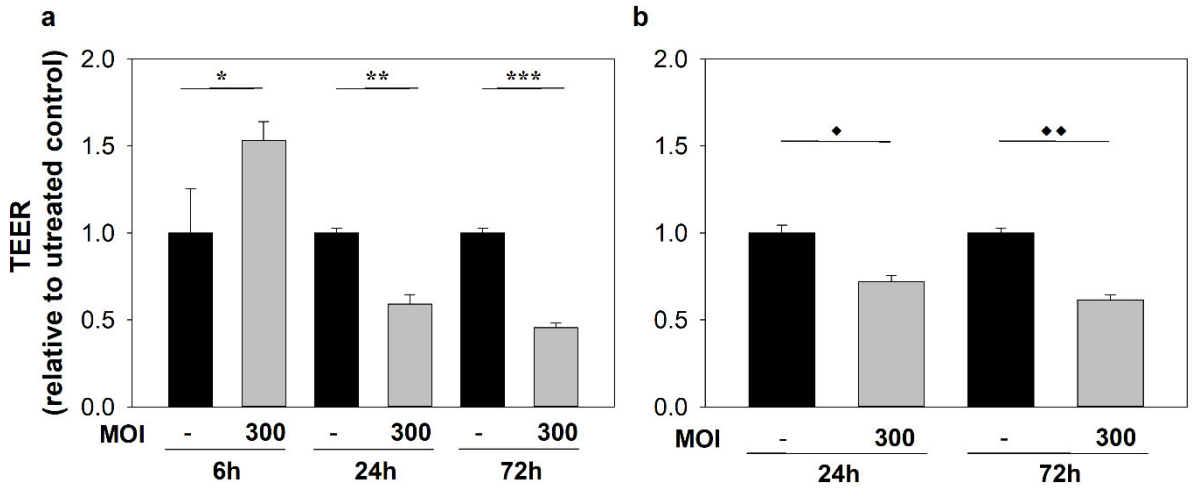


**Figure 8. Transepithelial electrical resistance (TEER) analysis of *C. acnes* 889 co-cultured Ca-low (a) and Ca-high (b) HPV-KER monolayer cultures.** *C. acnes* 889 strain (MOI= 300) induced marked TEER in the HPV-KER monolayer cultures, and these results were confirmed with real-time cellular analysis experiments. (Each treatment was performed in three technical replicates. Data points are the mean ± SEM. Statistical analysis with Student's t-test, * $p<0.05$, ** $p<0.005$, *** $p<0.00005$, ♦ $p<5x10^{-15}$, ♦♦ $p<5x10^{-24}$)

Altogether, these results strongly suggest that *C. acnes* can influence the state of the *in vitro* cultured keratinocyte barrier.

### 4.5. Dose-dependent nCi changes of HPV-KER monolayers upon co-culturing with various doses of the *C. acnes* 889 strain

We further analyzed the dose-dependent changes of *in vitro* cultured HPV-KER keratinocytes upon treatment with *C. acnes* 889 strain. Our results showed that in the presence of the higher dose (MOI=300) of the bacterium, the measured nCi values start to grow more rapidly compared to the samples containing a lower dose (MOI=100) of *C. acnes,* which was followed by a sharp decrease when the bacterium started to overgrow the keratinocyte cultures. Based on these results, we suggest that the extent and exact shape of the curves depend on the applied MOI of *C. acnes* (Fig. 9).

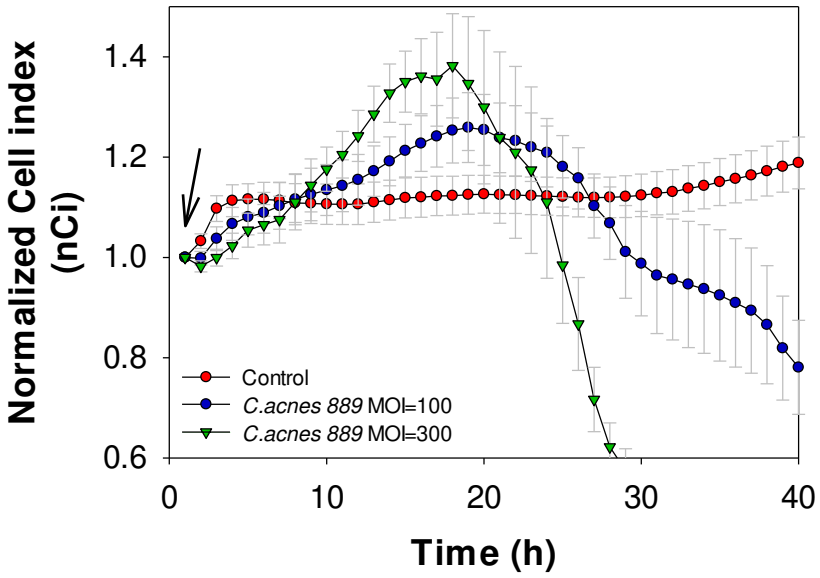


**Figure 9. In Ca-low HPV-KER monolayer cultures, co-culturing with *C. acnes* 889 strain leads to transient, dose-dependent nCi increases characteristic of improved barrier functions of *in vitro* monolayer cultures.** After the establishment of the Ca-low HPV-KER cultures, they were treated with different doses (MOI= 100, 300) of *C. acnes* 889 strain (0 hour time point, marked with an arrow). The transient, dose-dependent nCi increase observed is characteristic of improved barrier properties.

### 4.6. nCi changes of HPV-KER monolayers upon co-culturing with various *C. acnes* strains

For determining possible strain-specific effects, we repeated the bacterium treatments of HPV-KER and NHEK monolayers using different *C. acnes* strains (6609 and ATCC 11828). Our results showed that the measured nCi values changed in a similar manner that we detected with the *C. acnes* 889 strain: a transient increase of nCi values, characteristic of improved barrier properties was followed by a drop both in the Ca-low HPV-KER and NHEK cultures. Our results also showed that the exact timing of the nCi changes depended on the used *C. acnes* strain, the treatment dose, and the cell type (Fig. 10).

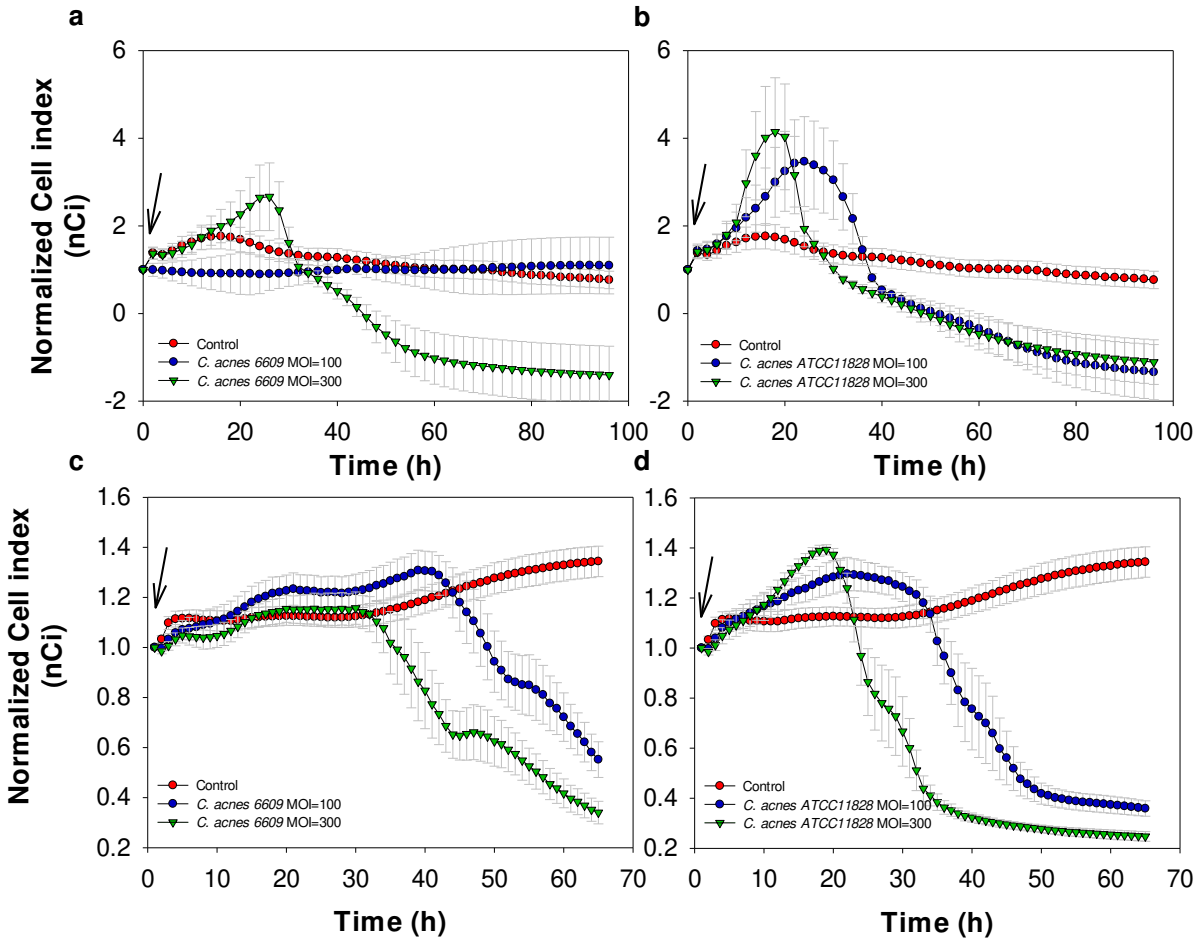


**Figure 10. Different *C. acnes* strains have variable effects on the nCi values of NHEK and HPV-KER monolayer cultures.** Ca-low NHEK (a, b) and HPV-KER (c, d) monolayer cultures were established and co-cultured with the *C. acnes* 6609 and ATCC11828 strains (0 hour time point, marked with an arrow) in different doses (MOI= 100, 300). Both strains lead to transient nCi changes similar to those observed for the *C. acnes* 889 strain, but with altered kinetics.

### 4.7. nCi changes of HPV-KER monolayers upon co-culturing with heat-killed *C. acnes* strains

We also investigated whether the observed cellular changes were dependent on the presence of a living bacterium. For this, we repeated our experiments using heat-killed *C. acnes* 889, 6609, and ATCC11828 strains. Our results showed no significant changes in the measured nCi values, suggesting that the impedance changes observed in previous experiments depended on the presence of live bacteria (Fig. 11).

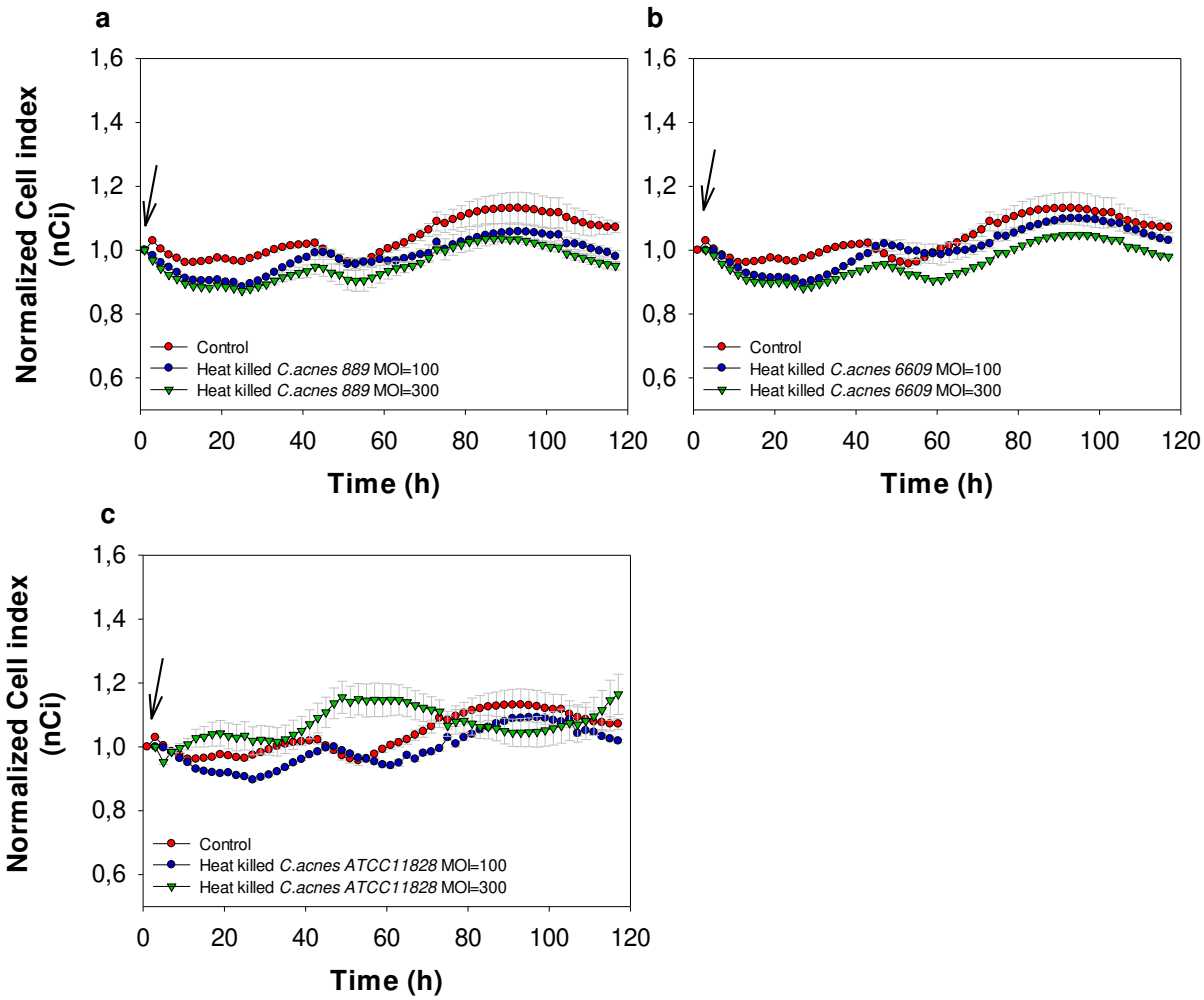


**Figure 11. Heat-killed *C. acnes* strains do not have major effects on Ca-low HPV-KER monolayer cultures.** After the establishment of contact-inhibited monolayer cultures, they were treated with heat-killed *C. acnes* 889 (a), 6609 (b), and ATCC11828 (c) strains (0 hour time point, marked with an arrow) using different doses (MOI= 100, 300). No major changes in nCi values were detected.

### 4.8. The effect of PA treatment on the barrier properties of *in vitro* cultured HPV-KER monolayer cultures

We also analyzed the effect of PA, which is an important molecule produced during the anaerobic fermentation processes of *C. acnes*, on the barrier properties of HPV-KER monolayer cultures. Our results showed that PA treatment alone has similar effects on the measured nCi values than that of live *C. acnes*. Indeed, treatments with high concentrations of PA resulted in a transient increase in nCi, followed by marked decreases suggesting that metabolic compounds produced by the *C. acnes* also affect the barrier properties of the *in vitro* cultured keratinocytes (Fig. 12).

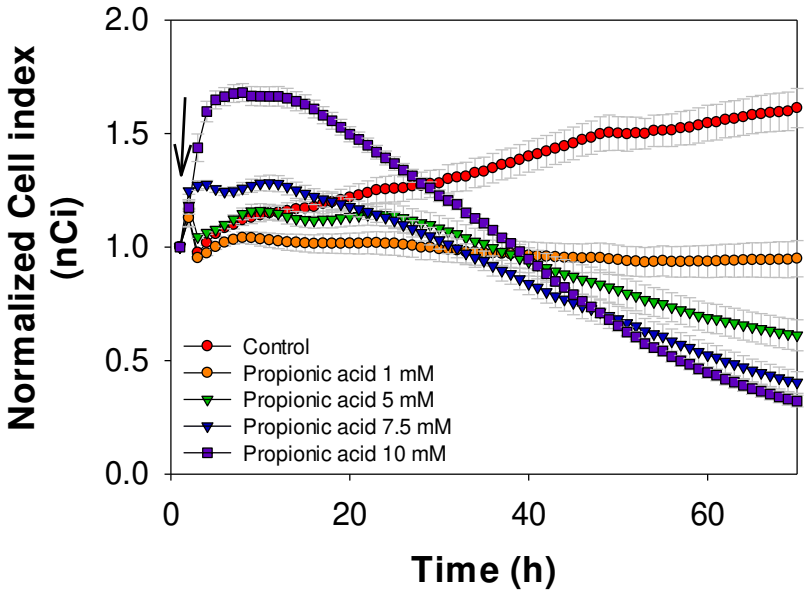


**Figure 12. PA treatment modulates the nCi values of Ca-low HPV-KER monolayer cultures.** Contact-inhibited monolayer cultures were treated with different doses of PA (1 mM, 5 mM, 7.5 mM, and 10 mM, 0 hour time point, marked with an arrow). Higher doses of PA treatment lead to transient nCi increases, characteristic of improved barrier properties of *in vitro* cultures.

### 4. 9. Expression changes of selected TJ proteins in HPV-KER monolayers upon co-culturing with *C. acnes* 889 strain

To study the cellular changes leading to *C. acnes*-induced nCi alterations, we treated Ca-low and Ca-high HPV-KER cultures with *C. acnes* 889 strain (MOI=100, 300) and analyzed the expression of selected genes encoding important TJ components: claudin 1 and 4 (CLDN1, CLDN4), occludin (OCLN) and zonula occludens 1 (ZO-1). mRNA expression studies were detected with real-time RT-PCR, and protein expression levels were detected with western analysis. ZO-1 mRNA levels did not markedly change, and OCLN expression increased upon bacterial treatment (Fig. 13 a, b; Fig. 15 a, b). The expression of the two claudins exhibited opposing changes: CLDN1 levels decreased in both models, while CLDN4 increased in the Ca-low cultures (Fig. 13 c, d, Fig. 15 c, d).

We found that, upon the addition of the bacterium, ZO-1 levels increased markedly in the Ca-high cultures (Fig. 16. a), and a small but consistent elevation was detected in the Ca-low HPV-KER cultures (Fig. 14. a). OCLN expression increased slightly (Fig. 14 b and Fig. 16 b), whereas CLDN1 protein levels decreased in both cultures (Fig 14 c and Fig. 16 c). CLDN4

levels also increased but only in the Ca-low cultures 72 hours after treatment (Fig 14. d and Fig. 16 d).

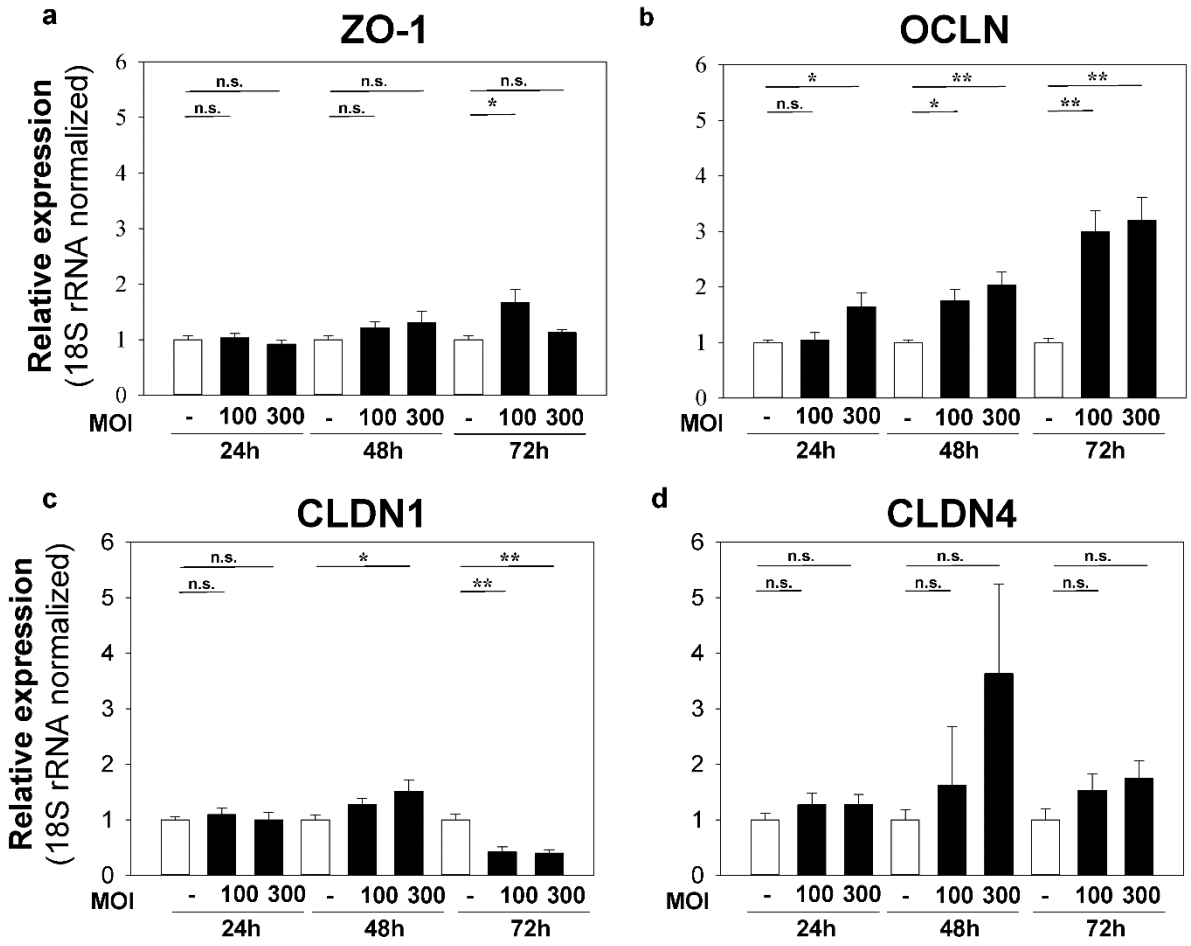


**Figure 13. mRNA expression changes of selected TJ proteins in Ca-low HPV-KER monolayer cultures co-cultured with *C. acnes* 889 strain.** ZO-1 levels did not change, and OCLN expression levels increased, while CLDN1 levels decreased upon bacterial treatment. CLDN4 mRNA levels showed a transient increase after *C. acnes* 889 treatment. (Data corresponds to the average of three parallel experiments, where each treatment was performed in three replicates. Data points represent the average ± SEM. Statistical analysis with Student's t-test, not significant (n.s.), * $p<0.05$, ** $p<0.005$.)

Our findings suggest that *C. acnes* induces the expression changes of selected TJ structural proteins.

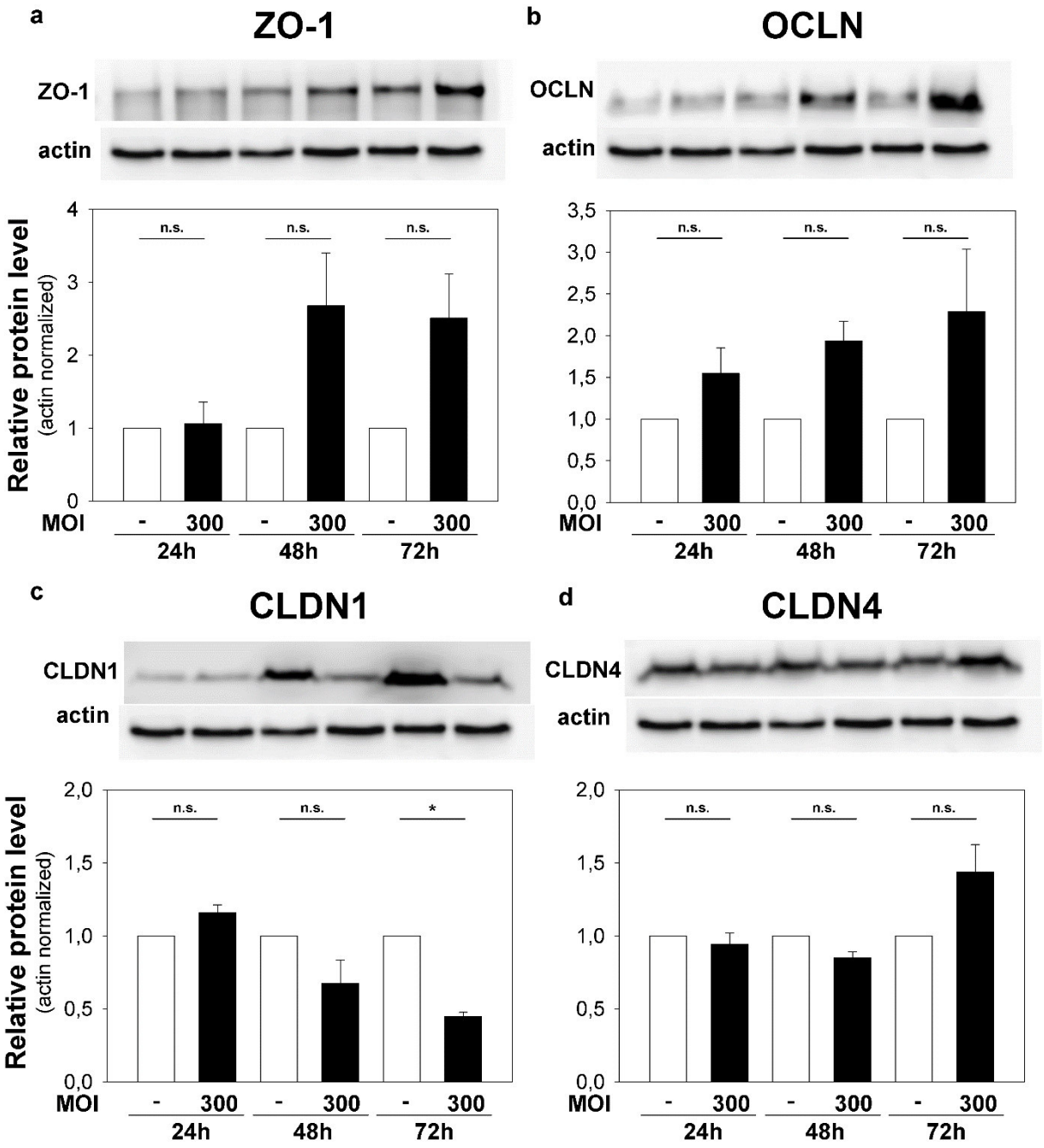


**Figure 14. Protein-expression changes of selected TJ proteins in Ca-low HPV-KER monolayer cultures co-cultured with *C. acnes* 889 strain.** ZO-1 and OCLN levels increased upon *C. acnes* 889 treatment. CLDN1 levels decreased, while in parallel to that, CLDN4 level increased upon bacterial treatment. (Data points represent the average ± SEM. Statistical analysis with Student's t-test, not significant (n.s.), * $p<0.05$ The representative western blot, and the graph correspond to the average of three independent experiments.)

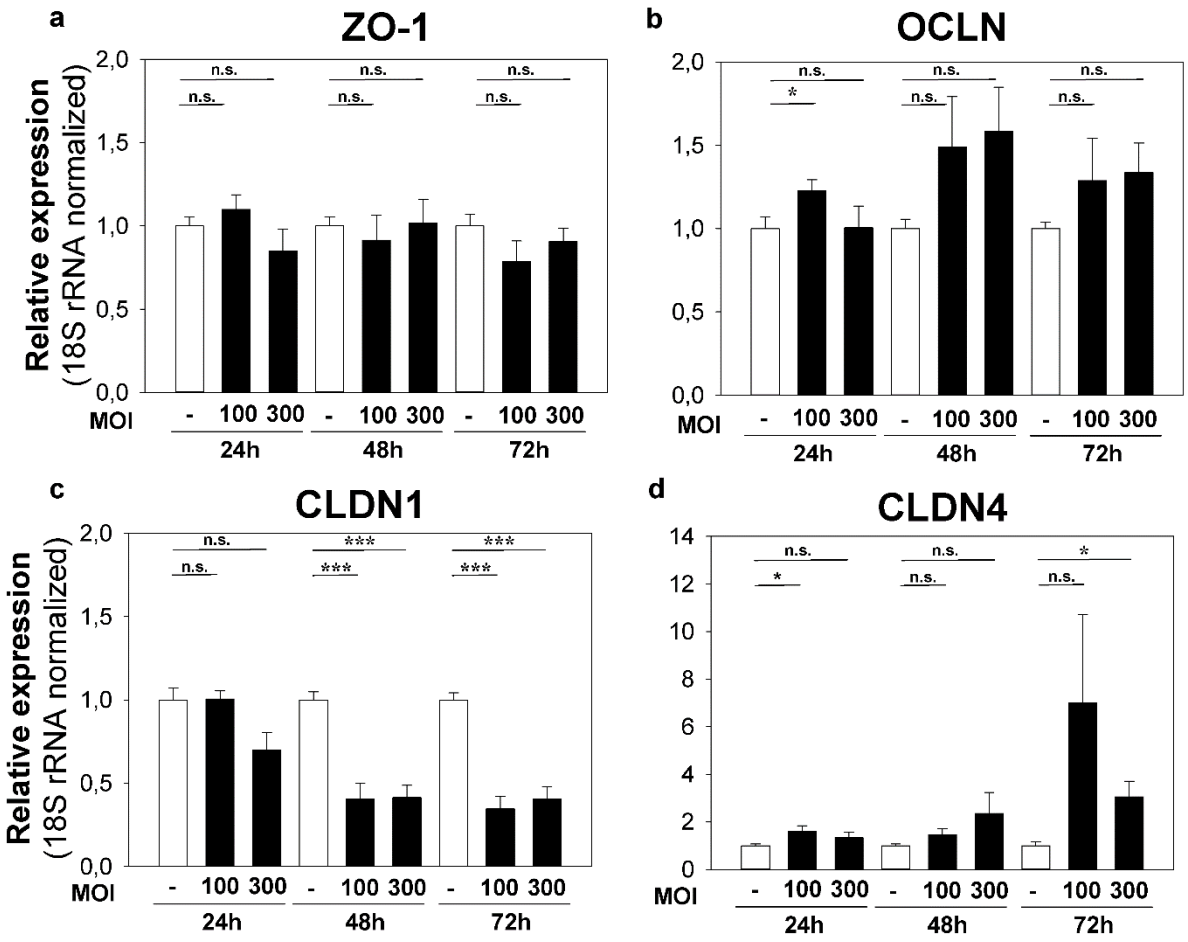


**Figure 15. mRNA expression changes of selected TJ proteins in Ca-high HPV-KER monolayer cultures co-cultured with *C. acnes* 889 strain.** ZO-1 levels did not show marked changes while OCLN expression increased upon bacterial treatment. CLDN1 level decreased while CLDN4 increased at the 72 hours samples upon *C. acnes* 889 treatment. (Data corresponds to the average of three parallel experiments, where each treatment was performed in three replicates. Data points represent the average ± SEM. Statistical analysis with Student's t-test, not significant (n.s.), * $p<0.05$, *** $p<0.00005$.)

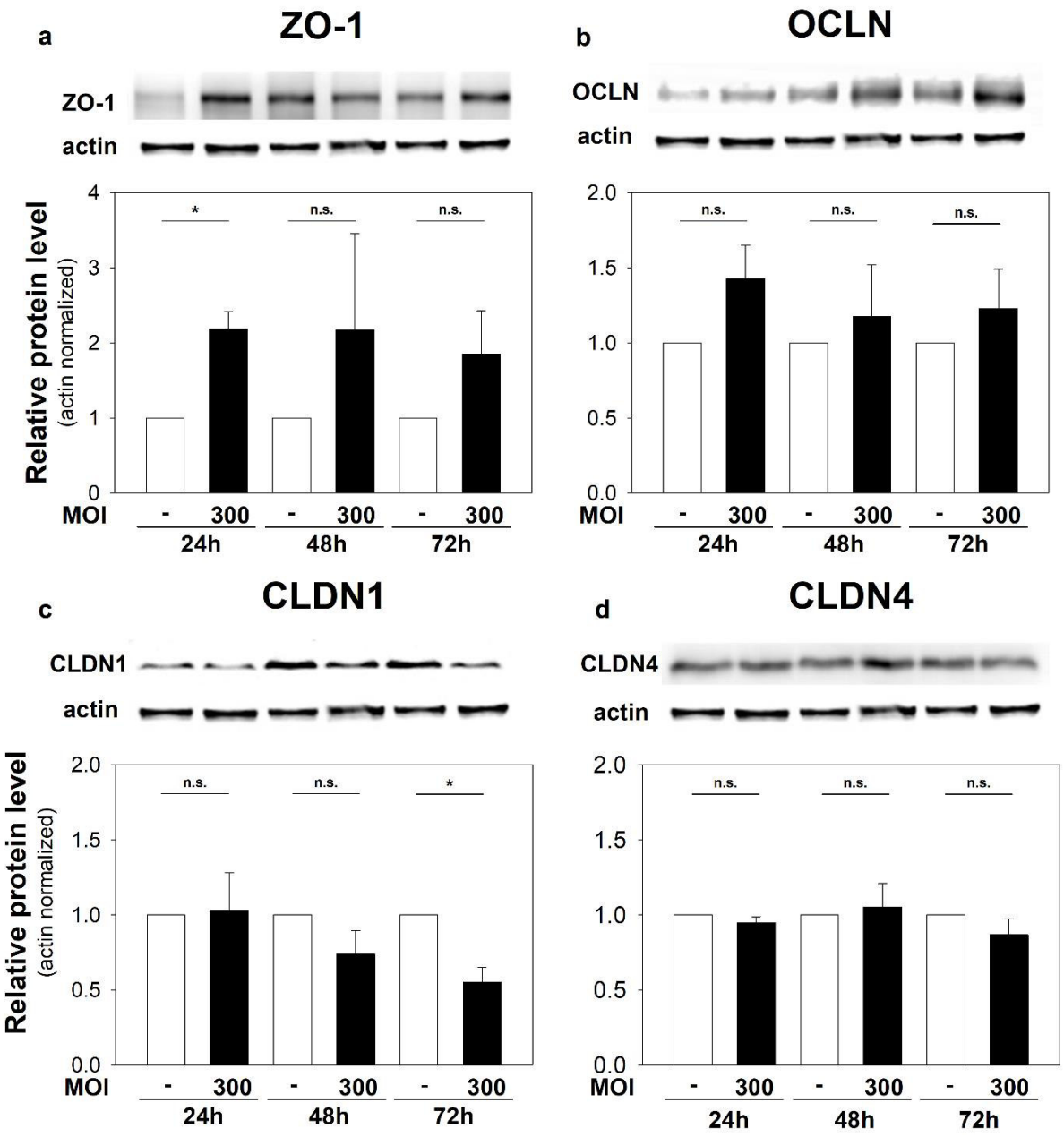


**Figure 16. Protein-expression changes of selected TJ proteins in Ca-high HPV-KER monolayer cultures co-cultured with *C. acnes* 889 strain.** ZO-1 and OCLN showed slightly elevated levels upon treatment with *C. acnes* 889 strain. CLDN1 levels decreased while CLDN4 did not change upon bacterial treatment. (Data points represent the average ± SEM. Statistical analysis with Student's t-test, not significant (n.s.), * $p<0.05$. The representative western blot and the graph correspond to the average of three independent experiments.)

### 4. 10. Expression changes of selected TJ proteins in OS models upon co-culturing with *C. acnes* 889 strain

To determine whether the *C. acnes*-induced cellular changes were specific to monolayer cultures, we also investigated the effect of the bacterium on the expression of selected TJ

structural components in *ex vivo*, three-dimensional OS cultures. In these experiments, we treated the upper, epidermal side of the skin samples with live *C. acnes* 889 strain. We collected samples at 24, 48, and 72 hours after treatment, and IHC staining was performed to monitor the mRNA and protein expression changes. We found that ZO-1 and OCLN protein levels increased in all epidermal layers in the presence of *C. acnes* 889 strain. CLDN1 expression, which was strongly expressed throughout the epidermis in the untreated control samples, decreased everywhere except the basal layer. In contrast, CLDN4, which was restricted to the *stratum granulosum* in control samples, also appeared in the lower layers of the epidermis in the presence of the bacterium (Fig 17.). These findings suggest that the abundance, as well as the localization of selected TJ proteins, change in the presence of the bacterium.

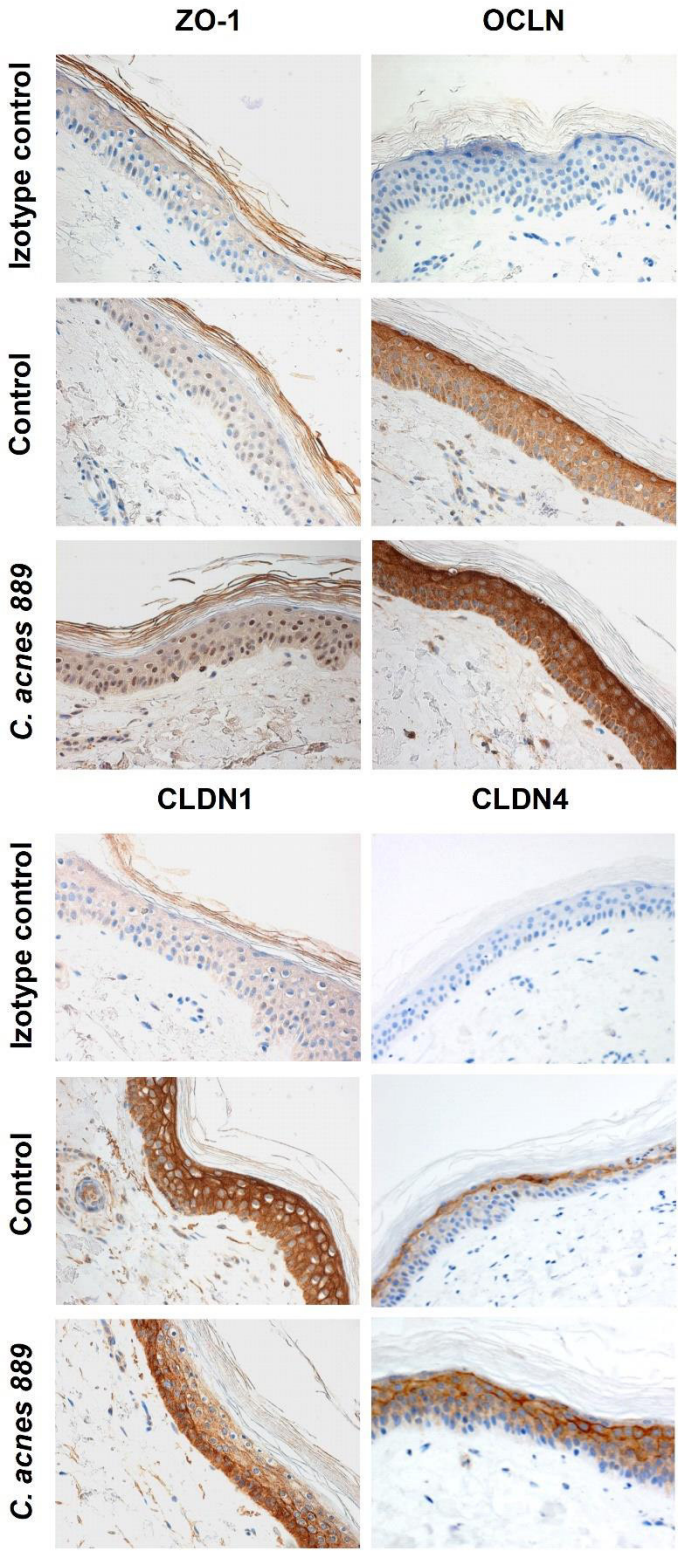


**Figure 17. TJ protein expression changes in OS models upon treatment with *C. acnes* 889 strain.** Full-thickness skin biopsy samples were treated with live *C. acnes* 889 strain (1.5 x $10^7$ bacteria/sample) for 72 hours. Paraffin-embedded sections were analyzed by IHC staining. We detected marked changes in expression and tissue localization for all the analyzed TJ proteins 72 h after bacterial treatment. (Representative image of three independent experiments.)

### 4. 11. LY penetration assay of HPV-KER monolayers and OS models upon co-culturing with *C. acnes* 889 strain

To analyze the functional consequences of the observed nCi changes, an LY penetration assay was performed on the Ca-high HPV-KER monolayers and OS models.

Ca-high cultures were first treated with *C. acnes* 889 strain (MOI=300) for 24 or 72 hours in a transwell system; controls were treated with PBS alone. We chose these time points based on the results of our previous TEER and xCELLigence experiments. LY dye was added to the upper chamber, and the penetration assay was carried out for 30 minutes. Samples from the cell supernatant collected from the lower chambers were subjected to fluorimetry.

We found that fluorescence intensities increased in treated samples compared to controls, indicating LY dye penetration by 72 hours after treatment. No differences were detected in the 24-hour samples (Fig. 18 a).

We also repeated the dye penetration experiments in the OS models to confirm that the observed barrier changes were not specific to monolayer cultures. *C. acnes* 889 strain or PBS was pipetted to the upper, epidermal side of the OS samples, and samples were incubated for 72 hours before the addition of LY and dye penetration was visualized by fluorescent microscopy. As observed for the monolayer cultures, LY dye penetration increased in the presence of high-MOI *C. acnes* 889 treatment, as indicated by the appearance of increased, diffuse fluorescent signal in the subepidermal skin regions (Fig. 18 b).

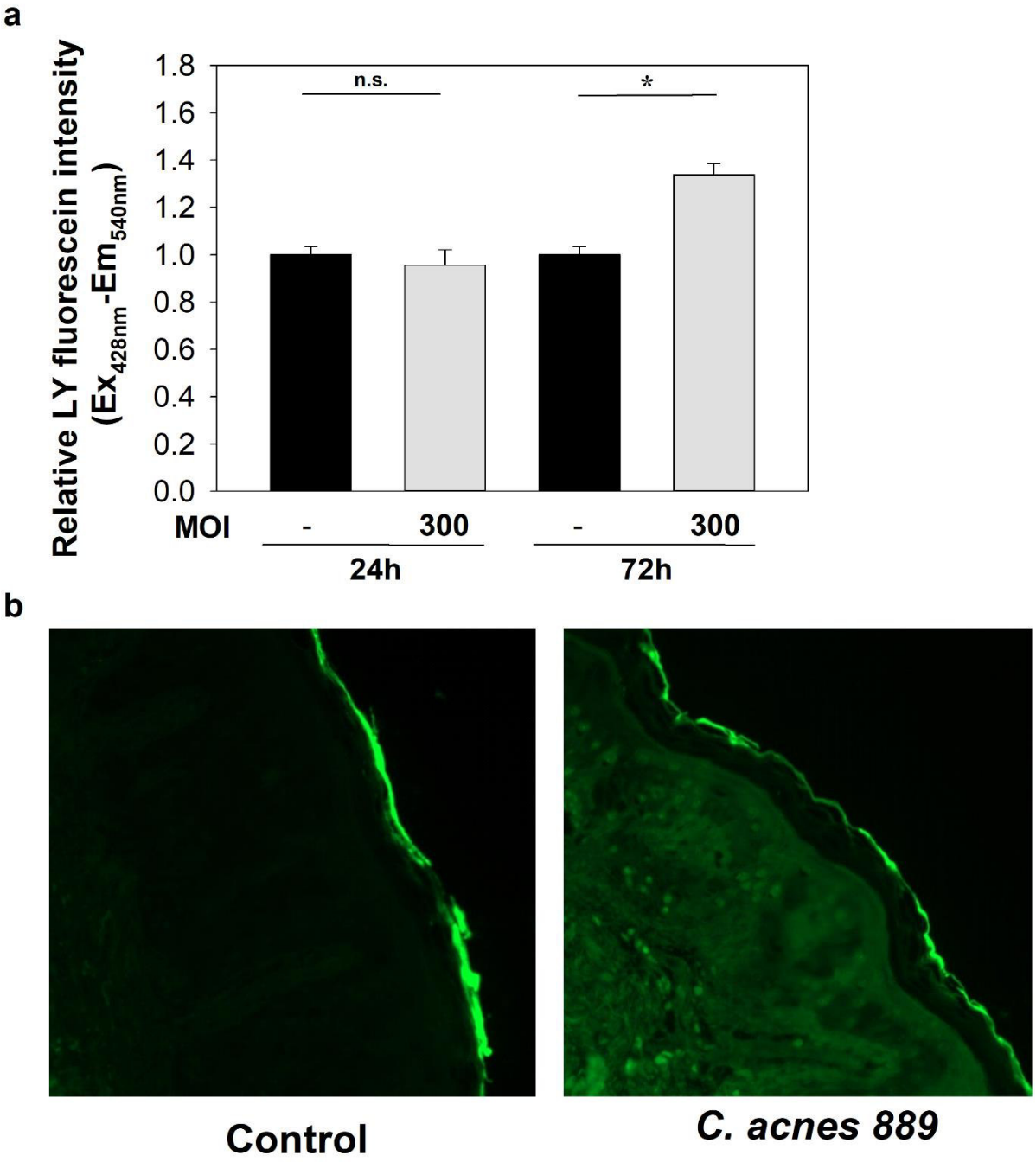


**Figure 18. LY dye penetration experiments.** Co-culturing with *C. acnes* 889 strain for 72 hours leads to increased LY dye penetration in Ca-high HPV-KER monolayer cultures (a) and OS models (b). (The graph represents the average of three technical replicates. Relative LY fluorescence intensity was compared to the time-matched untreated control samples. Data points represent the average ± SEM, statistical analysis with Student's t-test, not significant (n.s.), $p<0.0001$. Panel b is a representative of three independent experiments).

### 4. 12. Antibiotic treatment of HPV-KER monolayers co-cultured with *C. acnes* 889 strain leads to a partial reversal of bacterium-induced nCi decreases

As many of the acne treatments currently available exhibit bacteriostatic and/or antibacterial properties, we were interested to see if decreasing the *C. acnes* activity and/or viability in co-cultures with the addition of AB/AM treatment reverses the bacterium induced nCi decreases we noted in previous experiments. To this end, Ca-low HPV-KER cultures were co-cultured with *C. acnes* 889 strain (MOI=100), and the effects of the bacterium were continuously monitored in real-time using the xCELLigence system. When the nCi values started to drop, we changed the culturing media to KSFM supplemented with 1% or 5 % AB/AM solution. Decreasing the load of live bacteria in the co-cultures by the addition of AB/AM solution partially reversed the deleterious effect of *C. acnes* 889 strain and led to a marked increase of nCi values, characteristic of improved barrier functions of *in vitro* keratinocyte cultures (Fig. 19 a-e).

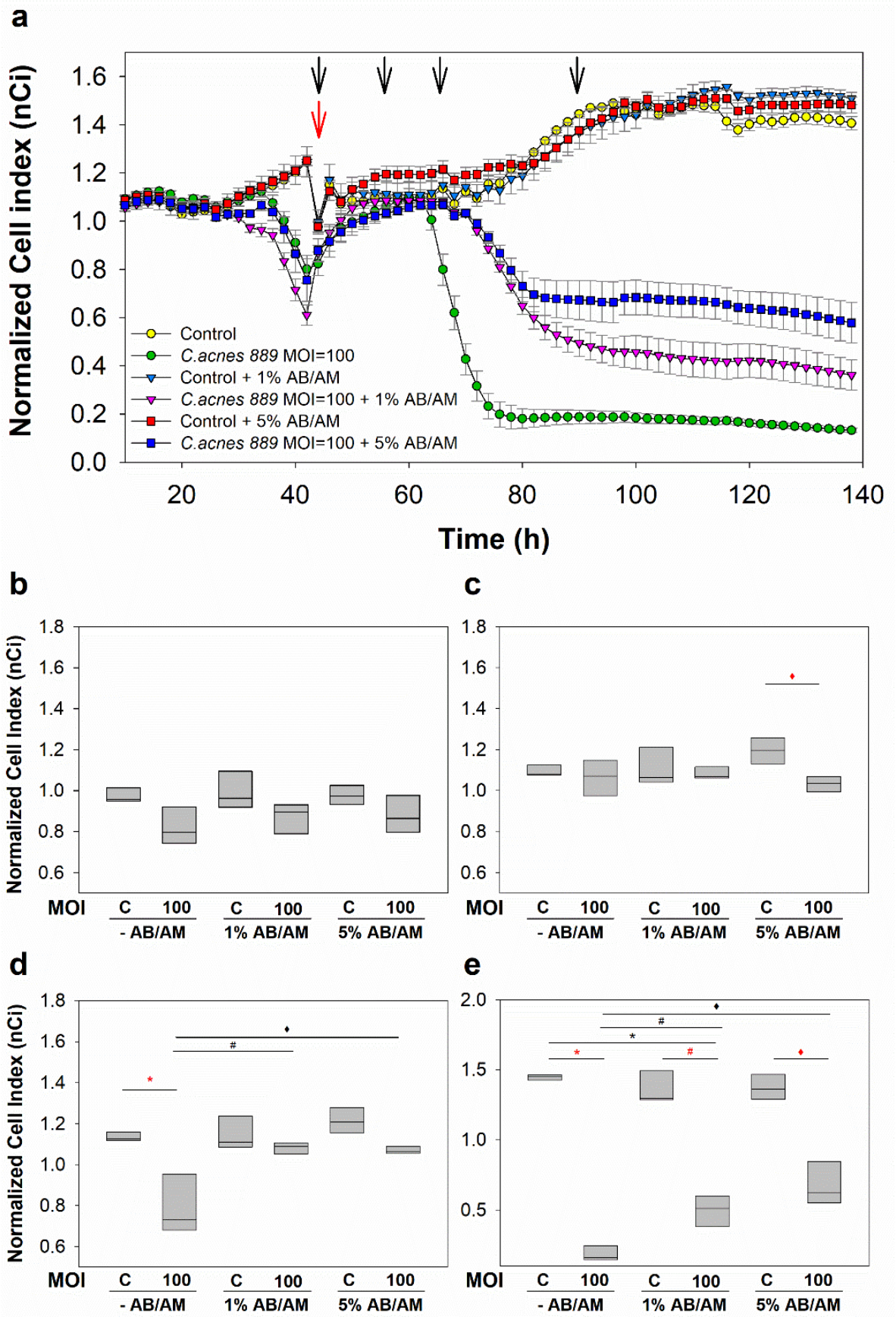


**Figure 19. Antibiotic treatment of *C. acnes* 889 co-cultured HPV-KER monolayers.** **(a)** Limiting the *C. acnes* bacterium with the addition of AB/AM solution transiently restores

the nCi values in a dose-dependent manner. 0h represents the time of *C. acnes* 889 treatment. (b-e) Box plot representation of selected time points after AB/AM treatment (red arrow) at 0 (b), 14 (c), 24 (d) and 48 hours (e) (marked with black arrows). (Representative image of three parallel experiments, where each treatment was performed in five technical replicates. Data points represent the mean ± SEM. Statistical analysis with one-way ANOVA, post-hoc Tukey test, $p<0.05$, alpha=0.05, * control *vs. C. acnes* 889, MOI=100, # control + 1% AB/AM *vs. C. acnes* 889, MOI=100 + 1% AB/AM, ♦ control + 5% AB/AM *vs. C. acnes* 889, MOI=100 + 5% AB/AM, * *C. acnes* 889, MOI=100 *vs. C. acnes* 889,MOI=100 + 1% AB/AM, # *C. acnes* 889, MOI=100 *vs. C. acnes* 889, MOI=100 + 5% AB/AM, ♦ *C. acnes* 889, MOI=100 + 1% AB/AM *vs. C. acnes* 889 MOI=100 + 5% AB/AM.)

## 5. Discussion

Although the cutaneous microbiota plays very important roles in the maintenance of the skin's homeostasis, the interactions between human cells and the various microbes also generate many challenges for the body. Skin cells, especially keratinocytes, must tolerate the presence of these microbes, even though they are equipped with all the conserved molecular structures that stimulate PRRs [32,46]. Thus, the skin must maintain a proper barrier against potentially harmful pathogens while simultaneously allowing continuous crosstalk between beneficial microbes and cutaneous immune cells [47–49]. Such complex interactions require fast, dynamic epidermal-barrier functions. In our study, we aimed to investigate whether and how the cutaneous microbiota, particularly the *C. acnes* bacterium affects the properties of the skin barrier.

Our interest led us to perform *in vitro* studies using two keratinocyte monolayer cultures (NHEK, HPV-KER) as a model system for skin with real-time impedance measurement-based studies. These methods are widely used for the detection of cellular integrity and barrier changes of *in vitro* monolayer cultures [39,50]. Using the gathered data, together with the results we obtained by analyzing full-thickness OS models, we propose a possible model, how *C. acnes* may affect the cutaneous barrier in the healthy skin and during acne pathogenesis.

We found that *C. acnes*, a prominent member of the skin microbiota, had a complex effect on these cells. In less differentiated, Ca-low cultures, co-culturing with live bacterium induced rapid, transient nCi increases, which are characteristic of improved barrier properties. These changes were strain-specific and depended on the applied bacterial MOI. A fast-growing pathogenic strain, *C. acnes* 889, IA, induced rapid and marked nCi increases compared to the *C. acnes* 6609 strain, which exhibits slower growth [35]. The effect of strain *6609*, which was originally isolated from the facial region of a healthy subject, exhibited a less pronounced, but sustained effect.

*C. acnes* is not the only microbe that may strengthen the skin barrier. Ohnemus and colleagues reported similar results with another member of the skin microbiota, *Staphylococcus epidermidis* [51]. Yuki *et. al.,* showed that TLR2 activation upon peptidoglycan (PGN) challenge enhanced the TJ barrier function in *in vitro* cultured keratinocytes and in human skin equivalents[52]. The observed phenomena are also very similar to those observed for the intestinal microbiota, which also aids the differentiation and the development of the anatomically mature, fully functioning gastrointestinal tract [53,54].

When left long enough in contact with the cells, continuously growing *C. acnes* strains gradually exhibited deleterious effects on the integrity and barrier properties of keratinocyte monolayer cultures, as indicated by the marked decrease in nCi values. This effect was dependent on the dose of live bacteria applied. The decrease in nCi was independent of cell differentiation. In addition, the change in nCi was not caused by keratinocyte death, as no major changes were detected in the number of live cells in the HPV-KER monolayer cultures.

nCi changes were only observed when we co-cultured the keratinocytes with live *C. acnes* strains. It is possible that these changes are not solely caused by conserved microbial structural components, but these changes might also involve a variety of special compounds that are generated by bacterial metabolism. *C. acnes* produces short-chain fatty acids as a result of anaerobic fermentation, enzymes (*e.g.,* lipases) and other biologically active molecules like Christie–Atkins–Munch-Peterson (CAMP) factors, that impact important regulatory functions of a number of human cell types [55]. As SCFAs and enzymes have an effect on the surrounding milieus like how they play a role in the modulation of the pH level of the skin, they may also function as pathogenic factors to cause harm in the barrier function of the tissue. CAMP factors are contributing to the pathogenicity of strains as these factors are responsible for example to the hemolytic effect of *C. acnes*. These can act as endotoxins and show a cytotoxic effect on the host cells, which may lead to barrier disruption in the affected area. Identifying which of the many molecules are necessary for the regulation of cutaneous barrier functions requires further investigation. As an initial effort, we analyzed the effect of PA, a special metabolic product of this bacterium that is linked to bacterial growth and metabolism [35]. We found that PA may be an important factor: Ca-low cultures treated with high doses of PA exhibited clear, transient Ci changes that were comparable to what we observed with live *C. acnes* strains.

Next, we aimed to see whether the detected resistance changes were correlated with changes in *in vitro* barrier integrity. Treatment with LY, a large molecular weight, fluorescently labelled compound that cannot freely permeate lipophilic barriers, clearly indicated that the nCi decrease could be the consequence of bacterium-induced deterioration of the integrity of the HPV-KER keratinocyte culture. This effect was not specific to monolayer cultures: similar results were found when the experiments were repeated in OS models, suggesting that *C. acnes* may also regulate cutaneous barrier properties, tightness and paracellular transport properties of the epidermis, even in a tissue environment.

Multiple different claudins, which are also essential proteins in TJ complexes, are present simultaneously in a given cell type, and the expression of various family members are highly context-dependent, controlled both spatially and temporarily. Many other factors,

including tissue type, age, differentiation state, external and intracellular signals and stimuli, are involved in their regulation [56,57]. The claudin content of a given cell is not stable and can change significantly in a matter of hours. Such continuous molecular remodelling, called switching, is an important process that allows the epidermal barrier to adapt to the ever-changing external and internal environments [18]. TEER and the associated paracellular permeability are thought to depend on the profile of different claudins expressed in a given cell type at a given point in time [58]. In the skin, keratinocytes express several different claudin family members, but the most abundantly present proteins are CLDN1, CLDN 4 and CLDN 7 [12]. It is interesting that apart from keratinocytes, sebocytes also express the same claudins. They play important roles in the regulation of a permeability barrier, and possibly also during the holocrine secretion process in these glands [59]. Whether and how *C.acnes* affects the expression of these claudins, and through that sebaceous gland functions requires further investigations.

Immunostaining of characteristic TJ proteins has been used to gain insight into the integrity of different barriers [39]. In full-thickness skin organ cultures, ZO-1 and OCLN protein levels increased in all epidermal layers following treatment with *C. acnes* 889 strain, and we observed similar changes in monolayer cultures. CLDN1 and CLDN4 expressions exhibited opposite changes: while CLDN1 levels mostly decreased in the presence of the bacterium, CLDN4, which was restricted to the *stratum granulosum* in control OS samples, simultaneously appeared in the lower layers of the epidermis. These protein expression changes correlated well with changes in mRNA expression, especially in the case of OCLN, CLDN1 and CLDN4. These findings suggest that TJ protein levels and their localization change in the presence of the bacterium, and their regulation possibly involves transcriptional and post-transcriptional effects, as well [60].

CLDN1 is a key determinant of epidermal barrier functions in human and mouse epidermis [61,62]. A strong positive correlation between epidermal integrity and CLDN1 expression levels was found in rodents. CLDN1-deficient mice exhibit signs of hyperkeratosis and acanthosis with aging, suggesting that abnormal regulation of this protein affects multiple cellular mechanisms [63]. Reduced CLDN1 levels appears to be a common theme in different chronic inflammatory skin diseases, including psoriasis, atopic dermatitis and rosacea [62–64].

CLDN4 levels increased when keratinocytes and cells of the OS model were co-cultured with *C. acnes* 889 strain. CLDN4 is also an important molecule of the epidermal barrier, and the decrease its expression in *in vitro* cultured keratinocytes has been associated with barrier defects. CLDN4 is considered a tightening claudin, and our results are compatible with the idea

that its upregulation presents a compensatory mechanism to restore barrier functions caused by decreased CLDN1 expression levels [65,66].

Opposing regulation of CLDN1 and CLDN4 has been reported before, and CLDN1/CLDN4 switching may be an important mechanism to regulate the epidermal barrier during inflammatory skin conditions [62,67].

Overall, through the induction of mostly TLR-dependent innate immune and inflammatory changes and the production of bioactive molecules, the *C. acnes* bacterium may induce innate immune and inflammation activation and autophagy, as well as altering the differentiation state of skin cells and epidermal barrier functions [32,46,68,69].

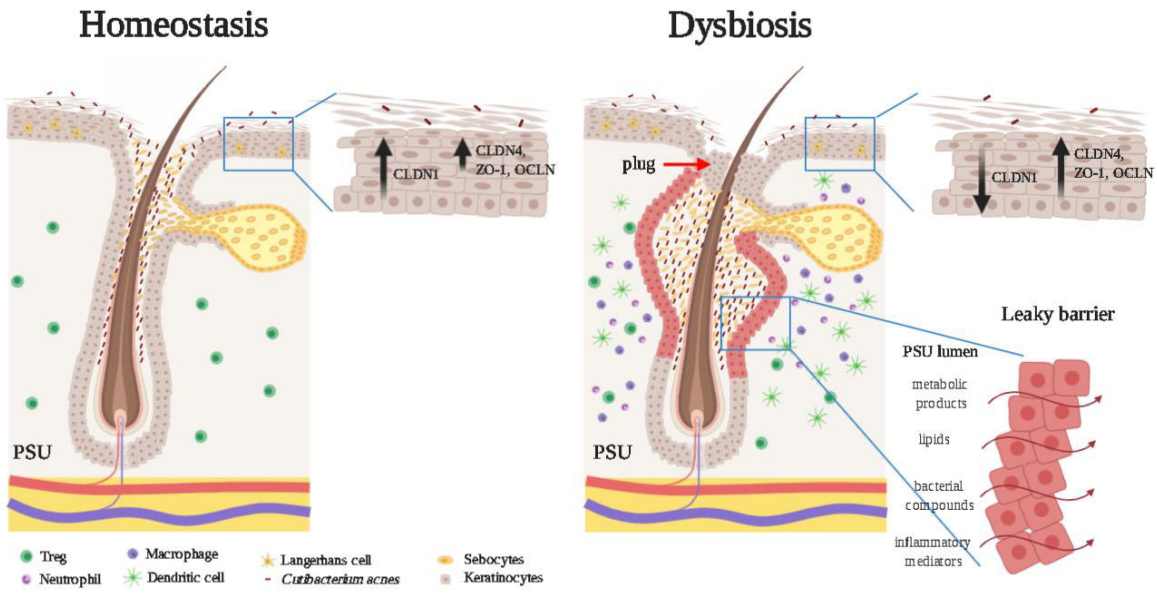


**Figure 20. Bacterial dysbiosis in the PSU leads to epidermal barrier changes in acne vulgaris pathogenesis.** During these events, the localization and expression of TJ structural components change leading to a leaky barrier. This allows the penetration of bacterial compounds, metabolic products, lipids, and inflammatory mediators to the deeper tissue layers. These results in the formation of more severe inflammatory lesions. (Schematic figure was created using the Biorender online software tool, http://www.BioRender.com)

In our experiments, the extent of barrier changes in keratinocyte cultures appeared to be strain- and dose-dependent: faster growing *C. acnes* strains (889 and ATCC11828) exhibited more pronounced changes. These results confirm our earlier findings, suggesting that, in parallel with bacterial growth, the pathogenic potential of *C. acnes* increases [35,68]. This conclusion contradicts findings that the amount of the bacterium does not differ in control and acne skin [70,71]. The reason for these discrepancies may be complex. Only a limited number of follicles are inflamed at any given time, and *C. acnes* may not reach the skin surface as it becomes trapped inside the follicles, especially when present in a biofilm form. Once inside the

pilosebaceous unit, its density may rapidly increase during lesion development. When this happens, this initially commensal bacterium may initiate a sequence of events and becomes pathogenic. In our studies, in the less differentiated, Ca-low HPV-KER cultures, co-culturing them with live bacterium induced rapid, transient nCi increases, which are characteristic of improved barrier properties. This effect may be the consequence of altered keratinocyte differentiation, as we detected similar Ci increases when we induced terminal differentiation in these cultures by raising the external $Ca^{2+}$ levels. These results are also in agreement with the findings of Akaza et al., showing that *C. acnes* affects the expression of various genes with a role in keratinocytes differentiation processes [6]. Abnormal regulation of keratinocyte proliferation and differentiation is a critical step in the induction of follicular hyperkeratinization during comedo formation in acne pathogenesis [72]. Terminally differentiated cells, together with the increased amount of sebum, may create a plug in the ductal region of the pilosebaceous unit. Below this occlusion, increasingly anaerobic conditions create a permissive environment for the anaerobic growth of the bacterium. These events go hand in hand with elevated production of bacterial molecules, including enzymes, SCFAs, and other potential virulence factors. The increasing level of innate and inflammation activation becomes apparent, leading to the development of more severe, inflammatory skin lesions, papules, pustules, and finally, cysts [72]. Our result suggests that parallel with these events, the state of the epidermal barrier within the follicles also changes (Fig. 20). While initially, *C. acnes* may help to develop and maintain a healthy keratinocyte barrier, its increasing presence and the growing concentration of molecules, including PA, within the follicle lumen start to have a negative effect. Through the deteriorating barrier, the transepithelial movement of bacterial antigens may increase, leading to inflammation in the surrounding tissues. In extreme conditions, the damage may become so severe that the increasing tension the retained sebum generates leads to the rupture of the follicle wall.

For acne treatment a large combination of different therapy methods can be used depending on the severity of the disease. For example physical treatment (lesion removal, phototherapy), pharmacological treatment (topical and/or systemic treatment)[73–75] and parallel to these adjuvant therapy also used (cleansing, moisturizing and sun protection) [76]. For mild acne topical therapy is applied, mostly benzoyl peroxide (BP), also topical retinoids and topical antibiotics or these in combination. For moderate and severe acne next to the topical therapy systemic therapy (oral antibiotics, hormonal therapy, and oral isotretinoin) is applied too in combination. The majority of these topical and systemic drugs exhibits direct or indirect bacteriostatic or antimicrobial properties which may modulate the bacterial load of the follicles

[73]. We tested whether reducing the presence of viable *C. acnes* using antibiotics in the HPV-KER co-cultures restores the barrier properties and found clear increases in the measured nCi values. Our results suggest that acne pathogenesis may be an even more complex event than previously suspected, and that, apart from the role of immune and inflammatory events, changes in cutaneous barrier properties can play important roles. Further experimental and clinical studies are necessary to determine whether therapeutic modalities restoring supplementary barrier together with conventional antibacterial treatments would enhance the healing of lesions.

## 6. New findings

1. *C. acnes*, a prominent member of the skin microbiota, have a complex, strain-specific, and dose-dependent effect on keratinocyte barriers. It improves barrier properties when present in low doses but may also exhibit deleterious effects due to its excess growth. The latter effect may play roles during lesion development in acne vulgaris pathogenesis.
2. Keratinocyte barrier changes are accompanied by altered expression and protein distribution of important TJ structural components, CLDN1, 4, OCLN, and ZO-1, leading to modified paracellular transport properties.
3. The barrier modifying effect of *C. acnes* is partly mediated by a short-chain fatty acid of anaerobic fermentation, propionic acid.
4. Antibiotics are widely used therapeutic modalities for acne vulgaris treatment. By decreasing the number of live bacteria in the diseased follicles, they may also aid the restoration of epidermal barrier functions.
5. Our results highlight the complex effect of *C. acnes* on the cutaneous barrier functions in healthy skin
6. We propose that acne pathogenesis may be an even more complex event than previously suspected, and apart from the role of *C. acnes* induced immune and inflammatory events, changes in cutaneous barrier properties can play important roles.

## 7. Conclusion

The human skin provides not only a physical or mechanical, but a chemical, microbiological, and immunological barrier, too. The entanglement of these barriers and the importance of a strictly regulated balanced interface between our body and the environment is critical for our well-being. There is rising evidence that the microbiota can affect different aspects of our skin. For the formation of a healthy and balanced immune barrier, they inhibit the colonization and growth of potential pathogens, help to train the cutaneous immune system to fight back infections, provides antimicrobial peptides, and secretes free fatty acids to maintain the acidic skin milieu to generate a hostile environment for other microbes. In turn, keratinocytes also closely monitor the microbiota changes. Through their pattern recognition receptors (*e.g.,* TLRs), they can sense the actual state of the microbial community and provide adequate signals to the surrounding cells to organize a complex response.

*C. acnes* is a prominent member of the cutaneous microbiota, and keratinocytes can recognize them through the TLR 2 and 4 receptors. By the activation of these receptors, innate immune and inflammatory events are induced [77,78]. Apart from these well-known events, TLR signaling also affects a series of other cellular events, among those the expression and localization of TJ proteins. Through these functions, the bacterium may directly regulate the strength of cell-to-cell contacts, and transepithelial transport events. By also aiding keratinocyte terminal differentiation, the cutaneous microbiota may be an important factor in the establishment and maintenance of the healthy skin's physical barrier.

*C. acnes* can play major roles in the pathogenic events during acne vulgaris pathogenesis. Extensive immune activation, together with bacterium-induced deleterious barrier changes may modify the permeability barrier in a way that it provides an easier passage for immunogenic and irritative bacterial compounds to the deeper skin tissues, which events will ultimately cause the worsening of acne lesions.

Altogether, based on our results, we propose that *C. acnes* has a complex effect on the cutaneous barrier functions in healthy skin and during pathogenic conditions.

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# I.

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# *Cutibacterium acnes* regulates the epidermal barrier properties of HPV-KER human immortalized keratinocyte cultures

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**Our skin provides a physical barrier to separate the internal part of our body from the environment. Maintenance of complex barrier functions is achieved through anatomical structures in the skin, the stratified squamous epithelium specialized junctional organelles, called tight junctions (TJs). Several members of our microbial communities are known to affect the differentiation state and function of the colonized organ. Whether and how interactions between skin cells and cutaneous microbes, including *Cutibacterium acnes* (*C. acnes*), modify the structure and/or function of our skin is currently only partly understood. Thus, in our studies, we investigated whether *C. acnes* may affect the epidermal barrier using in vitro model systems. Real-time cellular analysis showed that depending on the keratinocyte differentiation state, the applied *C. acnes* strains and their dose, the measured impedance values change, together with the expression of selected TJ proteins. These may reflect barrier alterations, which can be partially restored upon antibiotic–antimycotic treatment. Our findings suggest that *C. acnes* can actively modify the barrier properties of cultured keratinocytes, possibly through alteration of tight cell-to-cell contacts. Similar events may play important roles in our skin, in the maintenance of cutaneous homeostasis.**

One of the most important properties of our skin is the complex barrier it provides to separate the internal part of our body from the environment, limiting contact with harmful chemicals, microbes, allergens and radiation[1–3]. The major building blocks of the skin barrier are the keratinocytes, which are capable of recognizing the ever-changing environmental conditions and mounting appropriate responses to maintain the integrity of the human body[4,5].

Maintenance of complex barrier functions is achieved through anatomical structures in the skin. The stratified squamous epithelium is the uppermost skin layer that contains live keratinocytes and contains specialized junctional organelles, called tight junctions (TJs), which are localized between the cells of the second and third layer of the stratum granulosum[6]. TJs provide intimate links between adjacent cells and play major roles in establishing the epidermal barrier, as well as act as important determinants of transepidermal transport[7–9]. The complex, multi-protein structure of TJs includes more than 40 proteins[10,11]. Claudin (CLDN) protein family members are some of the most important TJ components, as they are critical for the regulation of barrier functions, including permselectivity, which determines the size, ionic charge and electric resistance of molecules that may be transported through the barrier[12,13].

Keratinocytes are also in constant contact with various members of the cutaneous microbiota. One of the most well-known members of this community is the *Cutibacterium acnes* (*C. acnes*) bacterium, which, beginning with puberty, is a dominant species and preferentially inhabits sebum-rich skin regions[14,15]. Current research is elucidating a very interesting, mutualistic relationship between skin cells and this bacterium. The human skin offers a permissive environment for the colonization of *C. acnes* by providing nutrients, moisture, attachment

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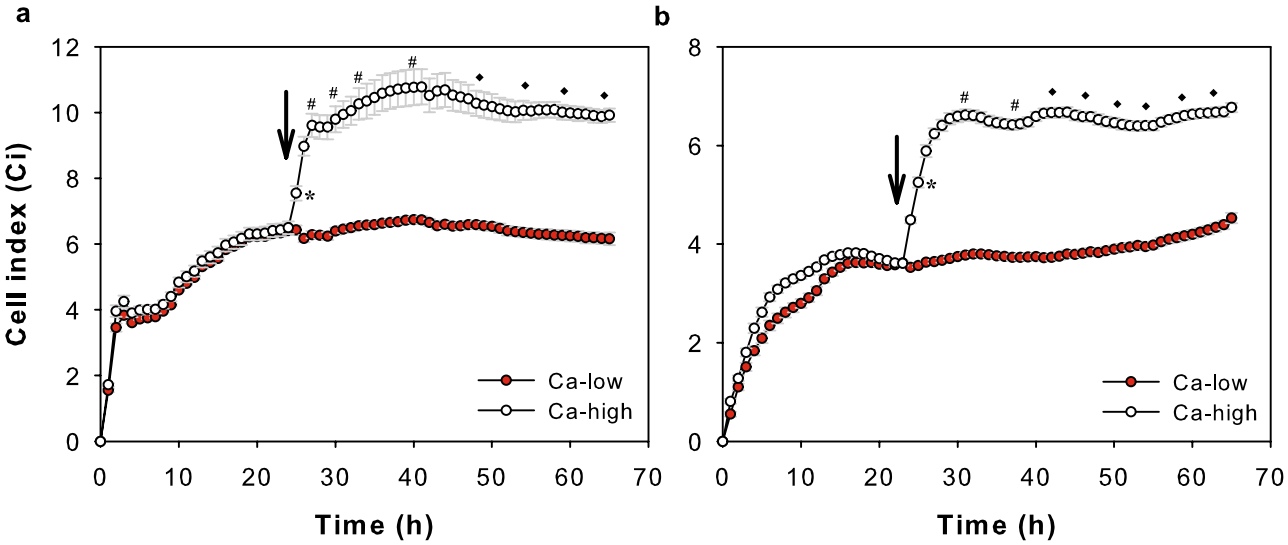



**Figure 1.** High $Ca^{2+}$ concentration leads to elevated Ci values of NHEK and HPV-KER cultures. Confluent NHEK (**a**) and HPV-KER (**b**) monolayer cultures were incubated in standard KSFM media. After 24 h (marked with an arrow), the extracellular $Ca^{2+}$ concentration was raised, leading to marked Ci increases. Representative image of three independent experiments. Data points are measured in every 4 h. Time points are the mean ± SEM. Statistical analysis with Student's t-test, $^{*}p < 0.05$, $^{\#}p < 0.005$, $\blacklozenge p < 0.0005$.

sites and optimal temperature for the growth of the bacterium. *C. acnes*, in return for these available resources, produces a hostile environment for other, potentially pathogenic microbes[16,17]. As a result, *C. acnes* is now viewed as an important factor in the establishment and maintenance of epidermal homeostasis[18].

Keratinocytes recognize the presence of *C. acnes* in their environment through the interaction of pattern recognition receptors (PRRs) expressed by the human cells and conserved molecules produced by microbes[19,20]. Consequently, innate immune and inflammation activation occurs, and autophagy may be induced[19–21]. Even though *C. acnes* is an important commensal bacterium, it can become an opportunistic pathogen during puberty as a result of microbial dysbiosis, in which it participates in the pathogenesis of a common inflammatory skin disease, acne vulgaris[22]. It has long been appreciated that immune and inflammatory events are crucial for acne lesion formation, and *C. acnes* may actively participate in these events; however, the exact sequence of pathogenic molecular events is still not known[23].

Several members of our microbial communities are known to affect the differentiation state and function of the colonized organ. These functions are especially well described for the gut microbiota, which aids the differentiation and the development of anatomically mature, fully functioning gastrointestinal tract[24,25]. Whether and how interactions between skin cells and cutaneous microbes, including *C. acnes,* modify the structure and/or function of our skin is currently only partly understood. Thus, in our studies we investigate the effect of *C. acnes* on properties of the epidermal barrier and have found that this bacterium, similarly to other commensal microbes, also has a profound effect on these cutaneous functions. Our data also indicate that acne pathogenesis is possibly even more complex than was previously suspected, and, in addition to immune and inflammatory changes, altered barrier properties may also contribute to the disease of acne.

## Results

**Effect of *C. acnes* on the integrity of in vitro keratinocyte cultures.** Ohmic resistance and impedance measurements across a wide spectrum of frequencies are considered as good indicators of the integrity of cellular barriers[26–29]. Thus, we investigated how different culturing conditions affect the measurable impedance (Z), and the calculated Cell index (Ci) values of confluent NHEK (Fig. 1a) and HPV-KER (Fig. 1b) cultures using real-time cellular analysis, and interpreted the changes as alterations in barrier properties of the in vitro monolayer cultures[27–29]. We allowed the cells to form confluent monolayers using basal KSFM and then raised the concentration of extracellular $Ca^{2+}$, which is known to induce keratinocyte differentiation[30]. We found that, after an initial growth phase, Ci values reached a plateau as the cultures became confluent and contact-inhibited. Replacing the media with fresh media containing high $Ca^{2+}$ concentrations (Ca-high) induced a marked and immediate increase in Ci compared to samples that were only contact inhibited and maintained in low $Ca^{2+}$ media (Ca-low). NHEK and HPV-KER cultures behaved similarly, suggesting that the latter cells may be used as a model to analyse keratinocyte barrier functions in monolayer cultures (Fig. 1a,b). These findings also agree with the available literature data and suggest that high extracellular $Ca^{2+}$ concentration leads to the stabilization of keratinocyte barriers. In addition, the capability of the xCELLigence RTCA system for real-time monitoring of these properties was confirmed[31,32].

We used the same experimental setup to analyse how the addition of *C. acnes* to the Ca-low and Ca-high NHEK and HPV-KER cultures affected keratinocyte barriers. For this, *C. acnes 889* strain (MOI = 100, 300) was added and nCi values were recorded in every 4 h for 95 h (Fig. 2).

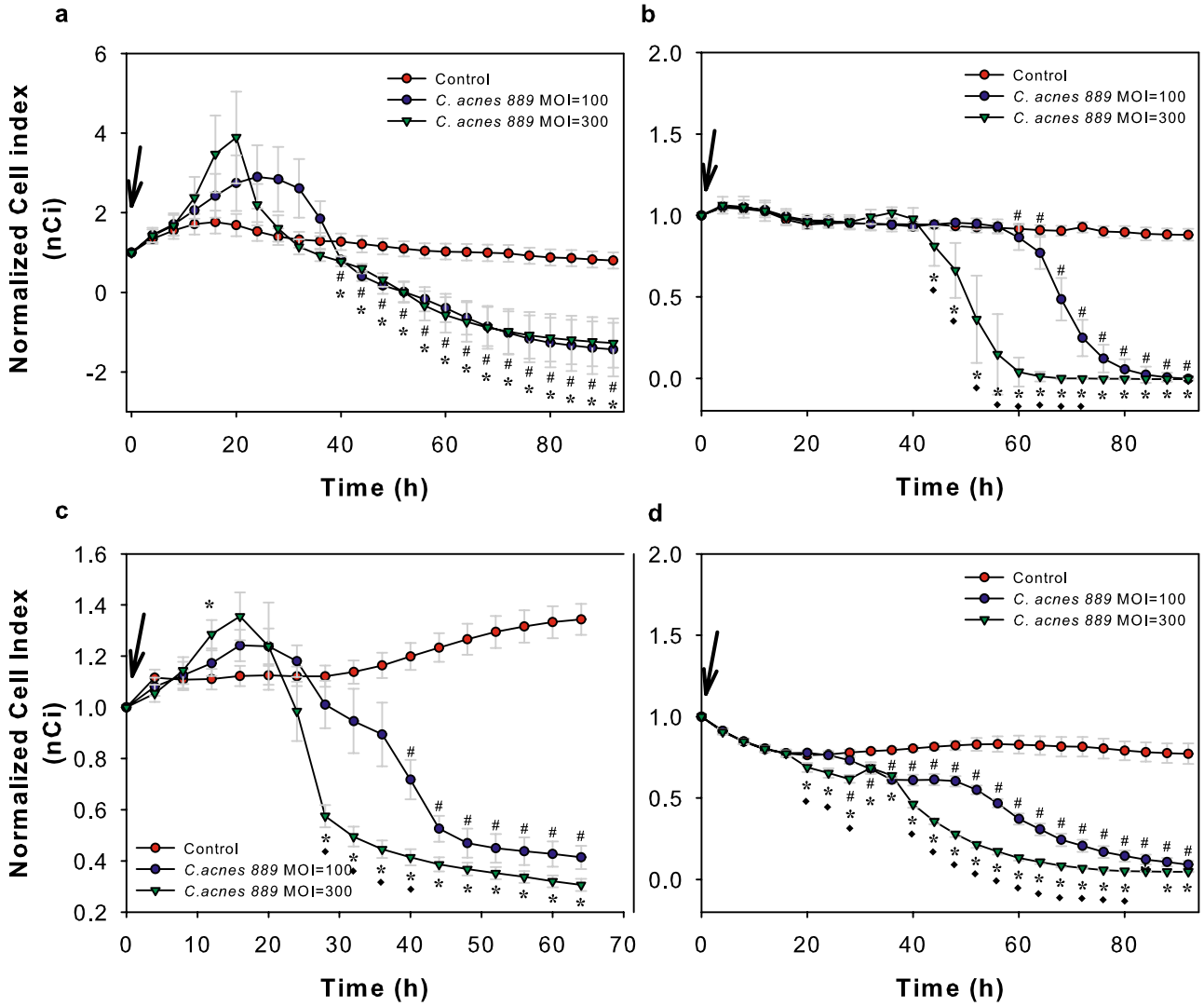



**Figure 2.** Co-culturing HPV-KER and NHEK keratinocyte monolayers with *C. acnes* 889 strain leads to dose-dependent nCi changes. Ca-low and Ca-high NHEK (**a**, **b**, respectively) and HPV-KER (**c**, **d**, respectively) keratinocyte monolayers were established and co-cultured with *C. acnes* 889 strain (0 h time point, marked with an arrow) in different doses (MOI = 100, 300). Addition of the bacterium induced dose-dependent nCi changes in all cultures. (For detailed description, see the corresponding text.) Data points are measured in every 4 h, representing the mean ± SEM. Statistical analysis with one-way ANOVA, post-hoc Tukey test, $p < 0.05$, alpha = 0.05, # control vs. *C. acnes 889,* MOI = 100, *control *vs. C. acnes 889*, MOI = 300, ♦ *C. acnes 889*, MOI = 100 *vs. C. acnes 889,* MOI = 300.

In Ca-low NHEK (Fig. 2a) and HPV-KER (Fig. 2c) cultures, we first observed a rapid and transient increase of nCi values, suggesting improved barrier properties of the cultures. Detailed analysis of these changes showed that the extent and exact shape of the curves depended on the MOI of *C. acnes* applied (Supplementary Fig. 1).

The peak in nCi was followed by a drop in the Ca-low cultures, suggesting that, after reaching a threshold, continuously growing bacterial cells may have deleterious effects on the barrier properties of keratinocyte monolayers. We also repeated the experiments on differentiated, Ca-high NHEK and HPV-KER monolayers. In these cell cultures only the dose-dependent decrease of nCi values were detected, independent of the used cell type and *C. acnes* MOI (Fig. 2b,d).

For determining strain-specific effects, we repeated the experiment using different *C. acnes* strains and HPV-KER monolayers. Similar results were detected, suggesting that the observed nCi changes may be independent of the applied strains (Supplementary Fig. 2).

When experiments were repeated using heat-killed *C. acnes*, no significant changes in nCi were detected, suggesting that the impedance changes observed in previous experiments depended on the presence of live bacteria (Supplementary Fig. 3a–c). This prompted us to test whether PA, an important molecule produced during *C. acnes* metabolism, produced similar effects. Indeed, treatments with high concentrations of PA resulted in a transient increase in nCi, followed by marked decreases (Supplementary Fig. 4).

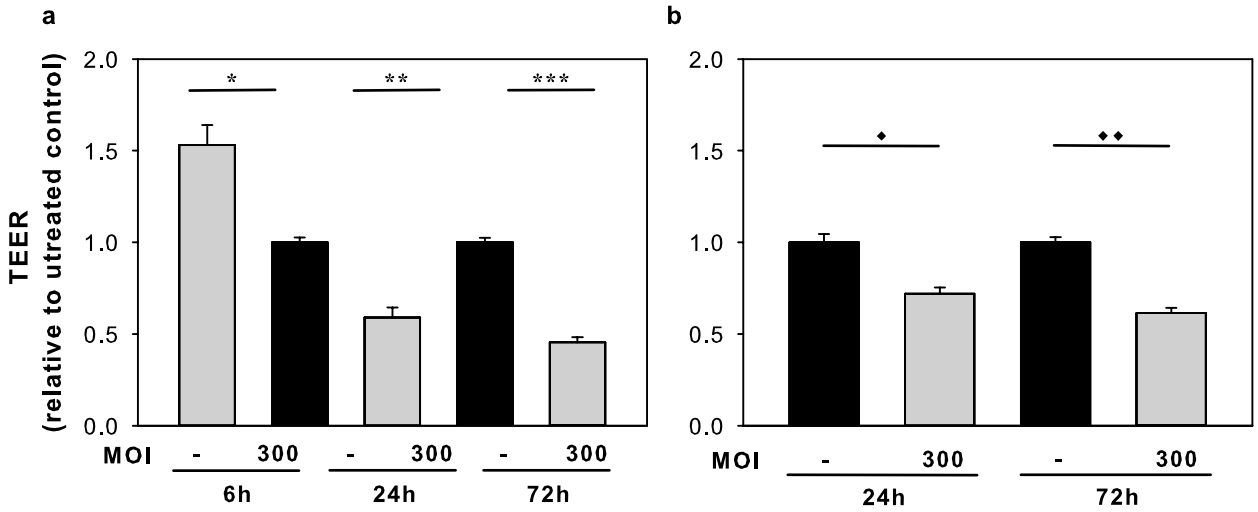



**Figure 3.** Transepithelial electrical resistance (TEER) analysis of *C. acnes 889* co-cultured Ca-low (**a**) and Ca-high (**b**) HPV-KER monolayer cultures. *C. acnes 889* strain (MOI = 300) induced marked TEER in the HPV-KER monolayer cultures, and these results were confirmed with real-time cellular analysis experiments. (Each treatment was performed in three technical replicates. Data points are the mean ± SEM. Statistical analysis with Student's t-test, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.00005$, ♦ $p < 5 \times 10^{-15}$, ♦♦ $p < 5 \times 10^{-24}$).

Apart from the barrier properties, Ci changes may reflect differences in the number or in the specific dimensions of cells attached to the electrodes. To determine the exact nature of the *C. acnes*-induced cellular events, we monitored the effect of the bacterium on the number of cells both in the Ca-low and Ca-high HPV-KER cell cultures. No significant change in cell number was detected using a trypan blue exclusion assay (Supplementary Fig. 5a,b).

We also performed TEER measurement, a widely accepted quantitative technique to analyse tight junction dynamics[26]. Selected time-points (24 and 72 h) were chosen, based on our real-time investigation, and TEER measurement was performed using untreated samples as a control and samples treated with *C. acnes* (MOI = 300) in both Ca-low and Ca-high HPV-KER monolayers. Our results indicate that the TEER resistance values first increased in the Ca-low cultures, followed by a decrease in both models in the presence of the bacterium, similarly to what we found in the xCELLigence analysis (Fig. 3).

Altogether, these results strongly suggest that *C. acnes* is able to influence the state of the in vitro cultured keratinocyte barrier. This effect requires the presence of metabolically active, live bacteria, and the extent of the changes are dependent on the bacterial dose.

**The effect of *C. acnes* 889 strain on the expression of selected TJ proteins in HPV-KER cultures.** To study the cellular changes leading to *C. acnes*-induced nCi alterations, we treated Ca-low and Ca-high HPV-KER cultures with *C. acnes 889* strain (MOI = 100, 300) and analysed the expression of selected genes encoding important TJ components: claudin 1 and 4 (CLDN1, CLDN4), occludin (OCLN) and zonula occludens 1 (ZO-1). mRNA expression studies were detected with real-time RT-PCR, and protein expression levels were detected with western analysis. ZO-1 mRNA levels did not markedly change, and OCLN expression increased upon bacterial treatment (Fig. 4a,b,i,j). The expression of the two claudins exhibited opposing changes: CLDN1 levels decreased in both models, while CLDN4 increased in the Ca-low cultures (Fig. 4c,d,k,l).

We found that, upon the addition of the bacterium, ZO-1 levels increased markedly in the Ca-high cultures (Fig. 4m), and a small, but consistent elevation was detected in the Ca-low HPV-KER cultures (Fig. 4e). OCLN expression increased slightly (Fig. 4f,n), whereas CLDN1 protein levels decreased in both cultures (Fig. 4g,o). CLDN4 levels also increased but only in the Ca-low cultures 72 h after treatment (Fig. 4h,p).

Our findings suggest that *C. acnes* induces the expression changes of selected TJ structural proteins.

**The effect of *C. acnes* 889 strain on the expression of selected TJ proteins in OS models.** To determine whether the *C. acnes*-induced cellular changes were specific to monolayer cultures, we also investigated the effect of *C. acnes* on the expression of selected TJ structural components in ex vivo, three-dimensional OS cultures. In these experiments, we treated the upper, epidermal side of the skin samples with live *C. acnes* 889 strain. We collected samples at 24, 48 and 72 h after treatment, and IHC staining was performed to monitor the mRNA and protein expression changes.

We found that ZO-1 and OCLN protein levels increased in all epidermal layers in the presence of *C. acnes* 889 strain. CLDN1 expression, which was strongly expressed throughout the epidermis in the untreated control samples, decreased everywhere except the basal layer. In contrast, CLDN4, which was restricted to the *stratum granulosum* in control samples, also appeared in the lower layers of the epidermis in the presence of the bacterium (Fig. 5). These findings suggest that the abundance as well as the localization of selected TJ proteins change in the presence of the bacterium.

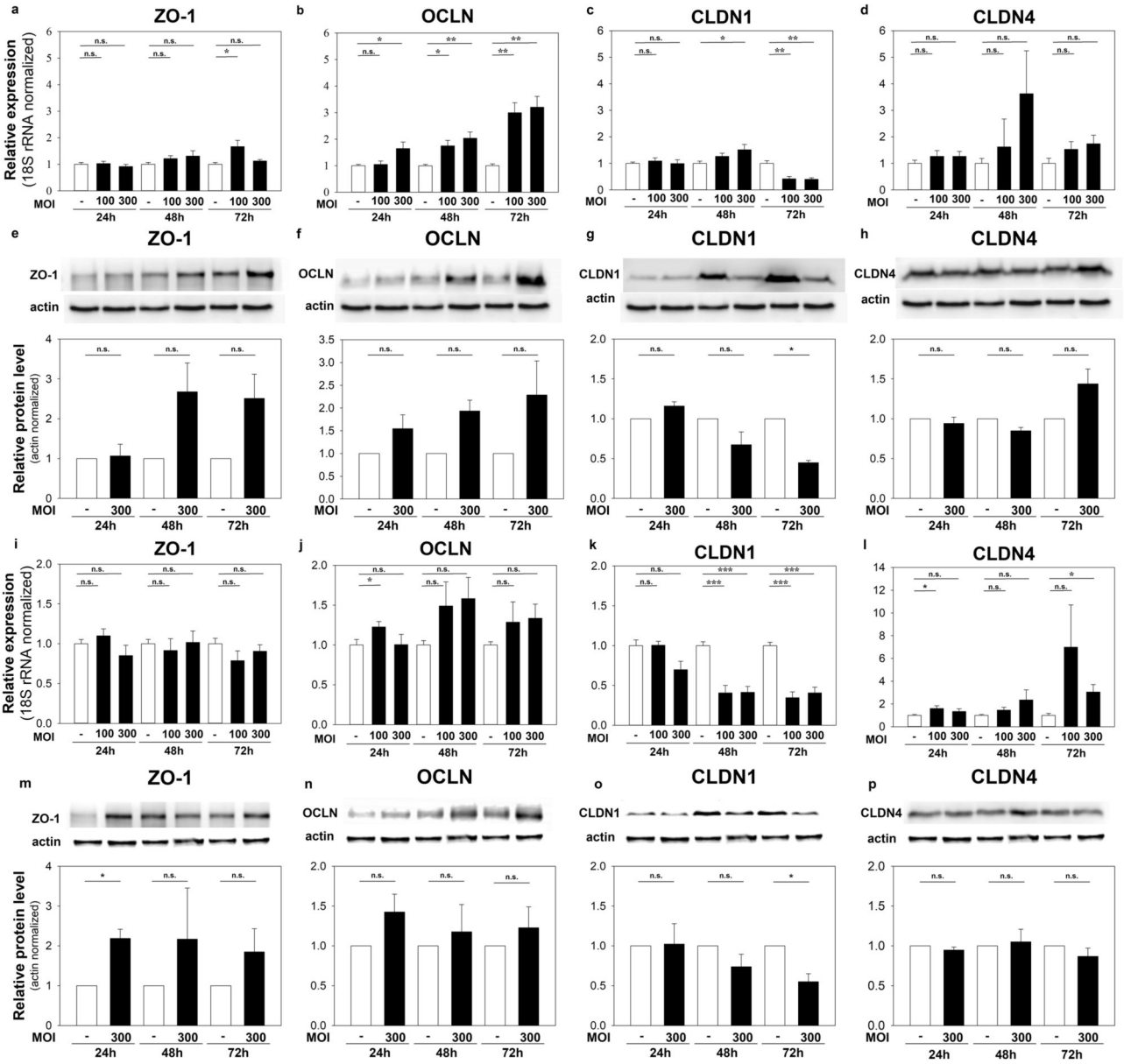



**Figure 4.** mRNA- and protein-expression changes of selected TJ proteins in Ca-low (**a**–**h**) and Ca-high (**i**–**p**) HPV-KER monolayer cultures co-cultured with *C. acnes* 889 strain. ZO-1 (**a**,**e**,**i**,**m**), OCLN (**b**,**f**,**j**,**n**), CLDN1 (**c**,**g**,**k**,**o**) and CLDN4 (**d**,**h**,**l**,**p**) mRNA and protein expression changes were monitored by real-time RT-PCR and western blot analysis. For detailed explanation, see the corresponding text. (mRNA data corresponds to the average of three parallel experiments, where each treatment was performed in three replicates. Western blot data shows a representative experiment. Detailed description can be find in Supplementary Figs. 6 and 7. Data points represents the average ± SEM. Statistical analysis with Student's t-test, not significant (n.s.), *$p < 0.05$, **$p < 0.005$, ***$p < 0.00005$. The representative western blot and the graph corresponds to the average of three independent experiments).

**LY penetration assay of HPV-KER monolayer cultures and OS models.** To analyse the functional consequences of the observed nCi changes, LY penetration assay was performed on the Ca-high HPV-KER monolayers and OS models.

Ca-high cultures were first treated with *C. acnes* 889 strain (MOI = 300) for 24 or 72 h in a transwell system; controls were treated with PBS alone. We chose these time points based on the results of our previous TEER and xCELLigence experiments. LY dye was added to the upper chamber, and the penetration assay was carried out for 30 min. Samples from the cell supernatant collected from the lower chambers were subjected to fluorimetry.

We found that fluorescence intensities increased in treated samples compared to controls, indicating LY dye penetration by 72 h after treatment. No differences were detected in the 24-h samples (Fig. 6a).

We also repeated the dye penetration experiments in the OS models to confirm that the observed barrier changes were not specific to monolayer cultures. *C. acnes* 889 strain or PBS was pipetted to the upper, epidermal

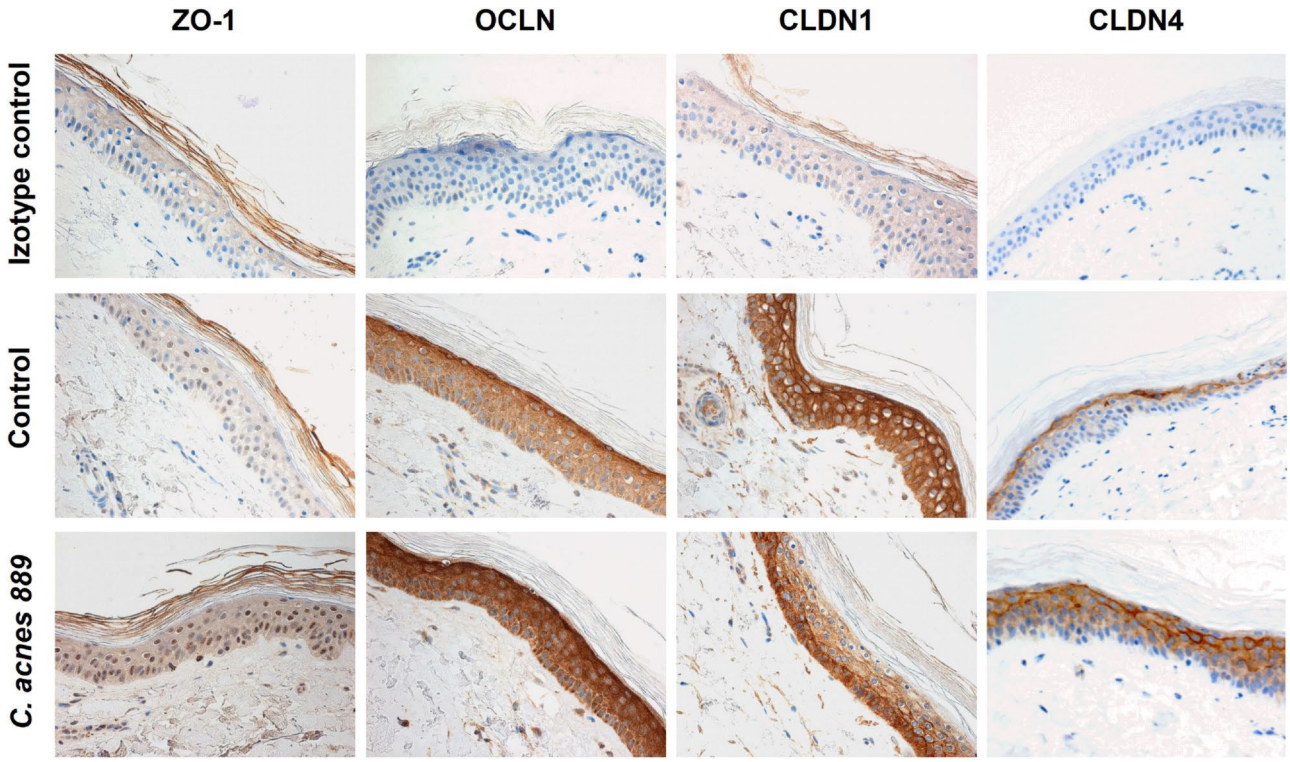



**Figure 5.** TJ protein expression changes in OS models upon treatment with *C. acnes* 889 strain. Full-thickness skin biopsy samples were treated with live *C. acnes* strain 889 ($1.5 \times 10^7$ bacteria/sample) for 72 h. Paraffin-embedded sections were analysed by IHC staining. We detected marked changes in expression and tissue localization for all the analysed TJ proteins 72 h after bacterial treatment. (Representative image of three independent experiments).

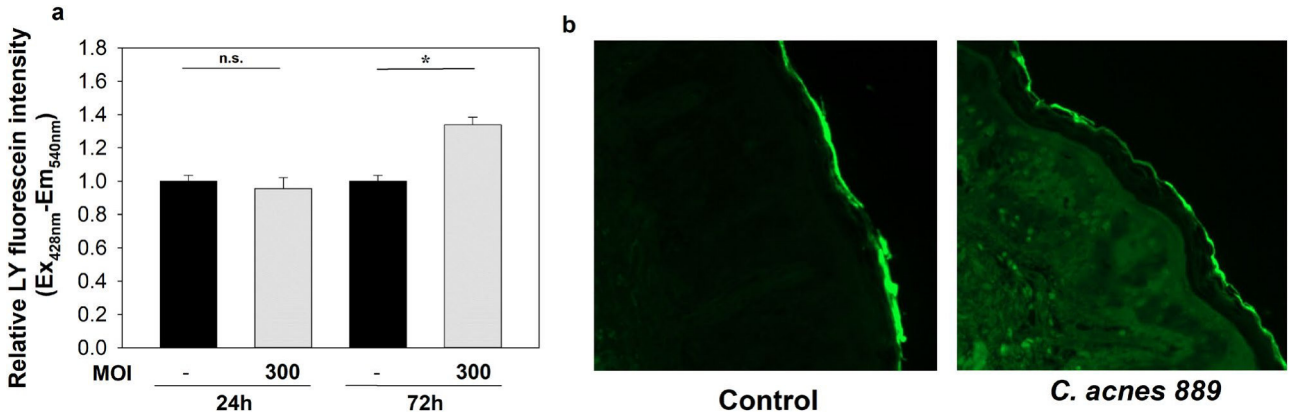



**Figure 6.** LY dye penetration experiments. Co-culturing with *C. acnes* 889 strain for 72 h leads to increased LY dye penetration in Ca-high HPV-KER monolayer cultures (**a**) and OS models (**b**). (The graph represents the average of three technical replicates. Relative LY fluorescence intensity was compared to the time-matched untreated control samples. Data points represent the average ± SEM, statistical analysis with Student's t-test, not significant (n.s.), $p < 0.0001$. Panel b is a representative of three independent experiments).

side of the OS samples and samples were incubated for 72 h before the addition of LY, and dye penetration was visualized by fluorescent microscopy.

As observed for the monolayer cultures, LY dye penetration increased in the presence of high-MOI *C. acnes* 889 treatment, as indicated by the appearance of increased, diffuse fluorescent signal in the sub epidermal skin regions (Fig. 6b).

**Antibiotic treatment of *C. acnes* co-cultured HPV-KER cells leads to the partial reversal of decreased nCi values.** As many of the acne treatments currently available exhibit bacteriostatic and/or

antibacterial properties, we were interested to see if decreasing the *C. acnes* activity and/or viability in co-cultures with the addition of AB/AM treatment reverses the bacterium induced nCi decreases we noted in previous experiments. To this end, Ca-low HPV-KER cultures were co-cultured with *C. acnes* 889 strain (MOI = 100), and the effects of the bacterium were continuously monitored in real time using the xCELLigence system. When the nCi values started to drop, we changed the culturing media to KSFM supplemented with 1% or 5% AB/AM solution. Decreasing the load of live bacteria in the co-cultures by the addition of AB/AM solution partially reversed the deleterious effect of *C. acnes* 889 strain and led to a marked increase of nCi values, characteristic of improved barrier functions of in vitro keratinocyte cultures (Fig. 7a–e).

## .Discussion

Although the cutaneous microbiota plays very important roles in the maintenance of the skin's homeostasis, the interactions between human cells and the various microbes also generate many challenges for the body. Skin cells, especially keratinocytes, must tolerate the presence of these microbes, even though they are equipped with all the conserved molecular structures that stimulate PRRs[19,20]. Thus, the skin must maintain a proper barrier against potentially harmful pathogens while simultaneously allowing continuous crosstalk between beneficial microbes and cutaneous immune cells[33–35]. Such complex interactions require fast, dynamic epidermal-barrier functions. In our study we aimed to investigate whether and how the cutaneous microbiota, particularly the *C. acnes* bacterium affects the properties of the skin barrier.

Our interest led us to perform in vitro studies using two keratinocyte monolayer cultures (NHEK, HPV-KER) as a model system for skin with real-time impedance measurement-based studies. These methods are widely used for the detection of cellular integrity and barrier changes of in vitro monolayer cultures[26,36]. Using the gathered data, together with the results we obtained by analysing full thickness OS models, we propose a possible model, how *C. acnes* may affect the cutaneous barrier in the healthy skin and during acne pathogenesis.

We found that *C. acnes*, a prominent member of the skin microbiota, had a complex effect on these cells. In less differentiated, Ca-low cultures, co-culturing with live bacterium induced rapid, transient nCi increases, which are characteristic of improved barrier properties. These changes were strain-specific and depended on the applied bacterial MOI. A fast growing pathogenic strain, *C. acnes 889*; IA, induced rapid and marked nCi increases compared to the *C. acnes 6609* strain, which exhibits slower growth[37]. The effect of strain *6609*, which was originally isolated from the facial region of a healthy subject, exhibited a less pronounced, but sustained effect.

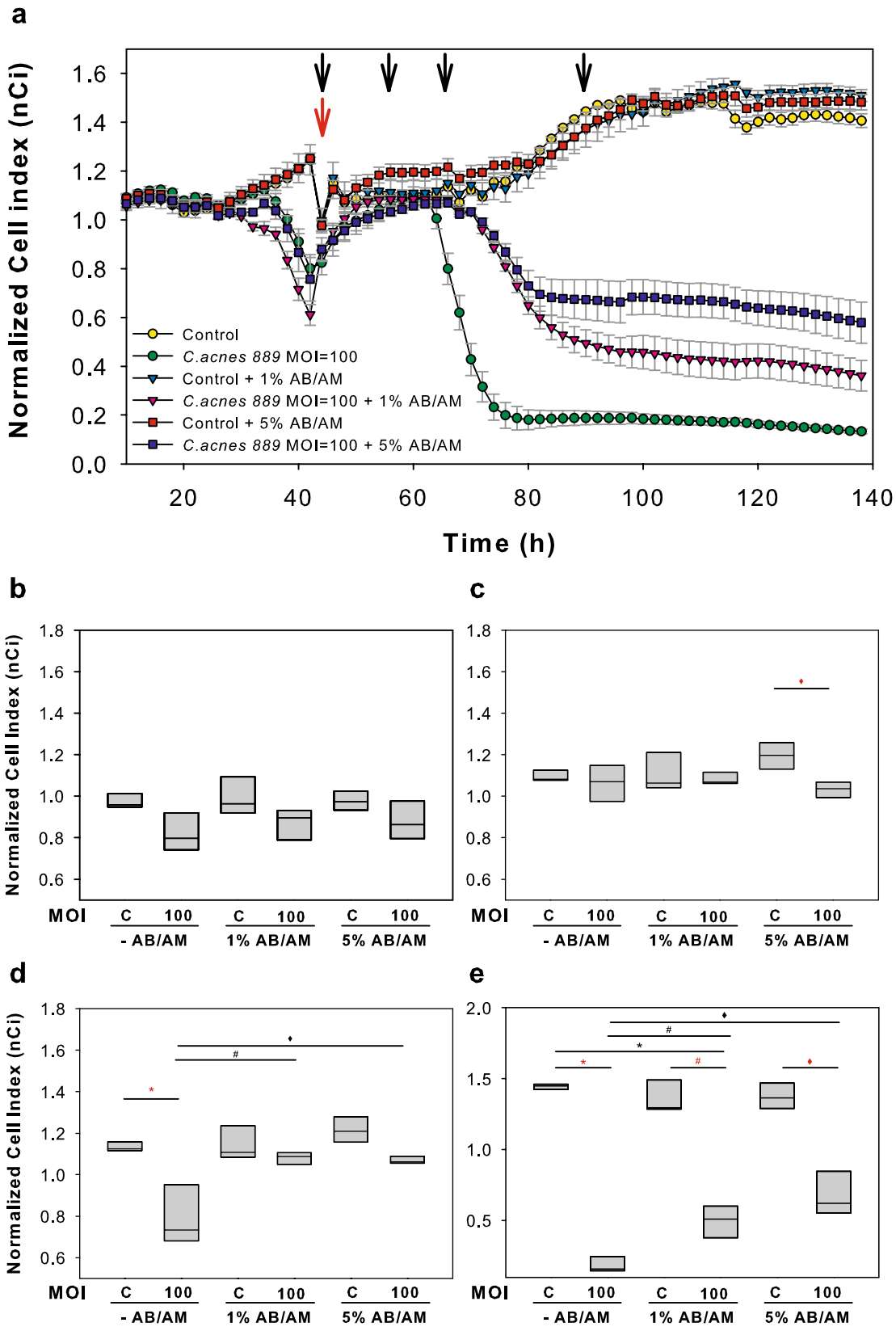
*C. acnes* is not the only microbe that may strengthen the skin barrier. Ohnemus and colleagues reported similar results with another member of the skin microbiota, *Staphylococcus epidermidis*[38]. The observed phenomena are also very similar to those observed for the intestinal microbiota, which also aids the differentiation and the development of the anatomically mature, fully functioning gastrointestinal tract[24,25].

When left long enough in contact with the cells, continuously growing *C. acnes* strains gradually exhibited deleterious effects on the integrity and barrier properties of keratinocyte monolayer cultures, as indicated by the marked decrease in nCi values. This effect was dependent on the dose of live bacteria applied. The decrease in nCi was independent of cell differentiation. In addition, the change in nCi was not caused by keratinocyte death, as no major changes were detected in the number of live cells in the HPV-KER monolayer cultures.

nCi changes were only observed when we co-cultured the keratinocytes with live *C. acnes* strains. It is possible that these changes are not solely caused by conserved microbial structural components, but these changes might also involve a variety of special compounds that are generated by bacterial metabolism. *C. acnes* produces short chain fatty acids as a result of anaerobic fermentation, enzymes (*e.g.,* lipases) and other biologically active molecules, that impact important regulatory functions of a number of human cell types[39]. Identifying which of the many molecules are necessary for the regulation of cutaneous barrier functions requires further investigation. As an initial effort, we analysed the effect of PA, a special metabolic product of this bacterium that is linked to bacterial growth and metabolism[37]. We found that PA may be an important factor: Ca-low cultures treated with high doses of PA exhibited clear, transient Ci changes that were comparable to what we observed with live *C. acnes* strains.

Next, we aimed to see whether the detected resistance changes were correlated with changes in in vitro barrier integrity. Treatment with LY, a large molecular weight, fluorescently labelled compound that cannot freely permeate lipophilic barriers, clearly indicated that the nCi decrease could be the consequence of bacterium-induced deterioration of the integrity of the HPV-KER keratinocyte culture. This effect was not specific to monolayer cultures: similar results were found when the experiments were repeated in OS models, suggesting that *C. acnes* may also regulate cutaneous barrier properties, tightness and paracellular transport properties of the epidermis, even in a tissue environment.

Multiple different claudins, which are also essential proteins in TJ complexes, are present simultaneously in a given cell type, and the expression of various family members are highly context-dependent, controlled both spatially and temporarily. Many other factors, including tissue type, age, differentiation state, external and intracellular signals and stimuli, are involved in their regulation[40,41]. The claudin content of a given cell is not stable and can change significantly in a matter of hours. Such continuous molecular remodelling, called switching, is an important process that allows the epidermal barrier to adapt to the ever-changing external and internal environments[42]. TEER and the associated paracellular permeability are thought to depend on the profile of different claudins expressed in a given cell type at a given point in time [12]. In the skin, keratinocytes express several different claudin family members, but the most abundantly present proteins are CLDN1, CLDN 4 and CLDN 7[43]. It is interesting that apart from keratinocytes, sebocytes also express the same claudins. They play important roles in the regulation of a permeability barrier, and possibly also during the holocrine secretion process in these glands[44]. Whether and how *C.acnes* affects the expression of these claudins, and through that sebaceous gland functions requires further investigations.


a
Normalized Cell index (nCi)
Time (h)
Control
C.acnes 889 MOI=100
Control + 1% AB/AM
C.acnes 889 MOI=100 + 1% AB/AM
Control + 5% AB/AM
C.acnes 889 MOI=100 + 5% AB/AM
b
c
d
e
Normalized Cell Index (nCi)
MOI
C
100
- AB/AM
1% AB/AM
5% AB/AM

◂**Figure 7.** Antibiotic treatment of *C. acnes* 889 co-cultured HPV-KER monolayers. (**a**) Limiting the *C. acnes* bacterium with the addition of AB/AM solution transiently restores the nCi values in a dose-dependent manner. 0 h represents the time of *C. acnes* 889 treatment. (**b**–**e**) Box plot representation of selected time points after AB/AM treatment (red arrow) at 0 (**b**), 14 (**c**), 24 (**d**) and 48 h (**e**) (marked with black arrows). (Representative image of three parallel experiments, where each treatment was performed in five technical replicates. Data points represent the mean ± SEM. Statistical analysis with one-way ANOVA, post-hoc Tukey test, $p < 0.05$, alpha = 0.05, red * control vs. *C. acnes 889,* MOI = 100, red # control + 1% AB/AM vs. *C. acnes 889,* MOI = 100 + 1% AB/AM, red ♦ control + 5% AB/AM vs. *C. acnes 889,* MOI = 100 + 5% AB/AM, * *C. acnes 889,* MOI = 100 vs. *C. acnes 889*,MOI = 100 + 1% AB/AM, # *C. acnes 889,* MOI = 100 vs. *C. acnes 889,* MOI = 100 + 5% AB/AM, ♦ *C. acnes 889,* MOI = 100 + 1% AB/AM vs. *C. acnes 889* MOI = 100 + 5% AB/AM).

Immunostaining of characteristic TJ proteins has been used to gain insight into the integrity of different barriers[26]. In full-thickness skin organ cultures, ZO-1 and OCLN protein levels increased in all epidermal layers following treatment with *C. acnes* 889 strain, and we observed similar changes in monolayer cultures. CLDN1 and CLDN4 expressions exhibited opposite changes: while CLDN1 levels mostly decreased in the presence of the bacterium, CLDN4, which was restricted to the *stratum granulosum* in control OS samples, simultaneously appeared in the lower layers of the epidermis. These protein expression changes correlated well with changes in mRNA expression, especially in the case of OCLN, CLDN1 and CLDN4. These findings suggest that TJ protein levels and their localization change in the presence of the bacterium, and their regulation possibly involves transcriptional and post-transcriptional effects, as well[45].

CLDN1 is a key determinant of epidermal barrier functions in human and mouse epidermis[46,47]. A strong positive correlation between epidermal integrity and CLDN1 expression levels was found in rodents. CLDN1-deficient mice exhibit signs of hyperkeratosis and acanthosis with aging, suggesting that abnormal regulation of this protein affects multiple cellular mechanisms[48]. Reduced CLDN1 levels appears to be a common theme in different chronic inflammatory skin diseases, including psoriasis, atopic dermatitis and rosacea[47–49].

CLDN4 levels increased when keratinocytes and cells of the OS model were co-cultured with *C. acnes* 889 strain. CLDN4 is also an important molecule of the epidermal barrier, and the decrease its expression in in vitro cultured keratinocytes has been associated with barrier defects. CLDN4 is considered a tightening claudin, and our results are compatible with the idea that its upregulation presents a compensatory mechanism to restore barrier functions caused by decreased CLDN1 expression levels[7,50].

Opposing regulation of CLDN1 and CLDN4 has been reported before, and CLDN1/CLDN4 switching may be an important mechanism to regulate the epidermal barrier during inflammatory skin conditions[47,51].

Overall, through the induction of mostly TLR-dependent innate immune and inflammatory changes and the production of bioactive molecules, the *C. acnes* bacterium may induce innate immune and inflammation activation and autophagy, as well as altering the differentiation state of skin cells and epidermal barrier functions[19–21,52].

In our experiments, the extent of barrier changes in keratinocyte cultures appeared to be strain- and dose-dependent: faster growing *C. acnes strains* (889 and ATCC 11828) exhibited more pronounced changes. These results confirm our earlier findings, suggesting that, in parallel with bacterial growth, the pathogenic potential of *C. acnes* increases[37,52]. This conclusion contradicts findings that the amount of the bacterium does not differ in control and acne skin[53,54]. The reason for these discrepancies may be complex. Only a limited number of follicles are inflamed at any given time, and *C. acnes* may not reach the skin surface as it becomes trapped inside the follicles, especially when present in a biofilm form. Once inside the pilosebaceous unit, its density may rapidly increase during lesion development.

Topical and systemic drugs are widely used for acne treatment[55]. We tested whether reducing the presence of viable *C. acnes* using antibiotics in the HPV-KER co-cultures restores the barrier properties and found clear increases in the measured nCi values. Our results suggest that acne pathogenesis may be an even more complex event than previously suspected, and that, apart from the role of immune and inflammatory events, changes in cutaneous barrier properties can play important roles. Further experimental and clinical studies are necessary to determine whether therapeutic modalities restoring supplementary barrier together with conventional antibacterial treatments would enhance the healing of lesions.

## Materials and methods

**C. acnes strains and culture conditions.** *C. acnes 889* (type IA) and *6609* (type IB) strains were cultured and stored as previously described in detail[20,37].

**Keratinocyte cell cultures, organotypic skin models and *C. acnes* treatment.** Normal human keratinocytes (NHEK) and ex vivo skin biopsies were obtained from skin specimens collected from the Plastic Surgery Unit of our department. Written, informed consent was obtained from the investigated individuals. The study was approved by the Human Investigation Review Board of the University of Szeged (PSO-EDAFN-002, 23 February 2015, Szeged, Hungary) and complied with the ethical standards of research in accordance with the Helsinki Declaration.

The human immortalized keratinocyte cell line HPV-KER[37] and NHEKs were used for our experiments. Both cell types 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 $CO_2$). Differentiation of confluent NHEK and HPV-KER cultures was induced with the addition of 1.7 mM $CaCl_2$.

For generating the organotypic skin (OS) models, 1 $cm^2$ whole-thickness skin samples were placed onto the nitrocellulose membrane of a 6-well insert, with the dermal surface placed on the membrane. Samples were cultured in an air–liquid interphase using Dulbecco's Modified Eagle's/F12 medium supplemented with 10% fetal bovine serum. *C. acnes* culture (15 µl, $10^9$ CFU/sample) was transferred to skin samples (epidermal surface) and further cultured for an additional 24, 48 or 72 h. Samples for immunofluorescent staining were fixed in 4% paraformaldehyde and embedded in paraffin. For PCR analysis, 6 mm punch biopsies were taken from the samples and the epidermis was separated from the dermis by incubating in dispase solution for 3 h at 37 °C. Epidermis samples were homogenized using the Ultra Turrax T8 homogenizer (IKA-WERKE). Total RNA was isolated using TRI-Reagent (Molecular Research Center; Cincinnati, USA).

**Real-time, label-free cellular analysis of keratinocyte cultures.** The integrity and the barrier properties of HPV-KER and NHEK monolayer cultures were detected in real-time using an impedance-measurement based technology (xCELLigence, Real Time Cell Analyser system, ACEA Biosciences, San Diego, USA)[31,56]. For contact inhibited cultures, NHEK and HPV-KER cultures were grown to confluency on fibronectin-coated 96-well E-plates (Ca-low cultures). After 24 h of growth, cells were co-cultured with live *C. acnes* strains belonging to different phylogenetic groups within the species (*889, ATCC 11828, 6609*) at various multiplicity of infection (MOI). Each treatment was performed in five technical replicates. Impedance (Z) values were measured every 60 min (unless otherwise noted), from which a dimension-free cell index (Ci) was calculated (Ci = [impedance at time point n – background impedance without cells]/nominal impedance value). In the used confluent cultures, Ci values depend on the applied cell number, the cell–cell adhesion and the cell-surface interaction. In some experiments, normalized Ci (nCi) values were also determined (nCi = Ci values at time point n/Ci values at a selected time point, *e.g.,* time point of applied treatment). Ci and nCi were plotted as a function of time. Each data point represents the mean ± the standard error of the mean (SEM).

For the differentiated NHEK and HPV-KER (Ca-high) cultures, KC-SFM culturing media was supplemented with 1.7 mM $CaCl_2$ after the cultures reached confluency. Forty-eight hours after treatment, the differentiated cultures were treated with *C. acnes* strains and nCi changes were monitored as described above.

The effect of propionic acid (PA) was determined using HPV-KER (Ca-low) cultures. After a confluent state was reached, PA was added to the cells at different concentrations, and nCi changes were monitored as described above.

Each treatment was performed in five technical replicates and the reported data points represent the mean ± SEM, unless otherwise noted.

**Western blot analysis of TJ proteins.** Cells were scraped with a cell scraper and harvested by centrifugation. The pellets were resuspended in protein lysis buffer (20 mM HEPES, 150 mM $KCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol, 5% TritonX-100, 10% glycerol, 0.1% NP-40) containing 1% protein inhibitor cocktail, 1% phenylmethylsulfonyl fluoride, 0.5% sodium dodecyl sulfate (all components from Sigma). Protein concentrations were measured with the BCA detection kit (Thermo Scientific, Waltham, MA, USA). SDS-PAGE was carried out using 50 µg protein sample, and proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with Tris-buffered saline solution (150 mM NaCl, 25 mM Tris, pH 7.4) containing 3% and 5% non-fat dry milk powder (Bio-Rad). Rabbit anti-human actin (Sigma-Aldrich) and anti-ZO-1 (Thermo Fisher Scientific) antibodies were diluted at 1:1,000, mouse anti-claudin 1 (Abnova), anti- occludin (Abnova) and anti-Claudin-4 (Thermo Fisher Scientific) were diluted at 1:500, and membranes were incubated with antibodies overnight at 4 °C. Anti-rabbit and anti-mouse IgG horseradish peroxides-conjugated (HRP-conjugated) secondary antibodies (SouthernBiotech) were applied, and the resulting bands were visualized using the C-DiGit Blot Scanner (LI-COR Biosciences, Nebraska, USA) or the Omega Lum G Chemidoc Imageing System (Aplegen, Inc, Pleasanton, CA, USA). Original western blots are provided as Supplementary Figs. 6 and 7.

**Total RNA isolation and real-time RT-PCR.** Total RNA was isolated from the monolayer cultures using TRI-Reagent (Molecular Research Center; Cincinnati, USA). cDNA synthesis was performed using EvoScript cDNA Synthesis Kit (Roche), and changes in the expression of TJ genes (CLDN1, CLDN4, OCLN and ZO-1) were analysed by real-time RT-PCR using the Universal Probe Library system (Roche, Indiana, USA). Supplementary Table 1 lists the PCR protocols and primer sequences used. TaqMan Gene Expression Assays (Thermo Fischer Scientific, Massachusetts, USA) were used to detect tumor necrosis factor α (TNFα; HS00174128_m1) and CXC motif chemokine ligand 8 (CXC8; HS00174103_m1). All data were normalized to the 18S rRNA using the $\Delta\Delta C_t$ method before comparing to the time-matched untreated control samples. Supplementary table 1 lists the used primers and probes.

**Immunohistochemical staining.** Paraffin-embedded OS samples were used to analyse changes in TJ protein levels after *C. acnes 889* treatment. A Bond-Max automated IHC/ISH stainer (Leica Biosystems, Wetzlar, Germany) was used for immunohistochemical (IHC) staining. The staining protocol is described here briefly. Six µm sections were cut, placed on glass slides, deparaffinized (dewax, 72 °C) and rehydrated. Subsequently, antigen retrieval was performed (10 mM citrate buffer, pH 6.0, 100 °C, 20 min). Primary antibodies against TJ proteins [CLDN1 (Abcam, Cambridge, UK), CLDN4 (Thermo Fischer Scientific, Massachusetts, USA), OCLN (Abnova, Taiwan), ZO-1 (Thermo Fischer Scientific, Massachusetts, USA)] were added to the samples at 1:100 dilutions (RT, 30 min), and also matching isotype controls [mouse IgG2a (Biolegend, CA, USA), rabbit poly IgG (Santa Cruz Biotechnology Inc, CA, USA) and mouse IgG1 κ (Biolegend, CA, USA)] which dilution rations were matched to the used primary antibodies concentrations. After primary antibody labelling, post primary steps

were performed for 8 min at room temperature (RT), subsequently, the polymer step was performed (15 min, RT). Peroxidase blocking was carried out (3 min, RT). Washing steps were performed before each step. Samples were developed with DAB-Chromogen for 10 min and dyed with hematoxylin. After covering with coverslips, samples were analysed with a microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with an AxioCam MRm camera.

**Transepithelial electrical resistance measurement.** Confluent HPV-KER monolayer cultures were grown on 12 mm transwell inserts (pore size 0.4 µm, Corning, New York, USA) in KSFM media. Confluent Ca-low cultures were treated with *C. acnes* 889 strain (MOI = 300) and transepithelial electrical resistance (TEER) values were measured in Hank's salt balanced solution (HBSS) using an Epithelial Volt/Ohm (TEER) Meter EVOM2 (World Precision Instruments, Sarasota, FL, USA) at 24 and 72 h after treatment.

For the Ca-high cultures, confluent, contact-inhibited keratinocyte cultures were further differentiated by the addition of $Ca^{2+}$ (1,7 mM) to the KSFM culturing media and incubation for 72 h. TEER values were measured at 24 and 72 h after treatment with *C. acnes* 889 in HBSS buffer using an Epithelial Volt/Ohm (TEER) Meter EVOM2 (Sarasota, FL, USA).

**Trypan blue exclusion assay.** HPV-KER cells were seeded on 12-well plates at a starting density of 300,000 cells/well. After 48 h, the cultures were co-cultured with various doses of *C. acnes* 889 strain (MOI = 100, 300). Samples were trypsinized and collected at 0, 24, 48 and 72 h after treatment, washed with phosphate-buffered saline (PBS) and stained with trypan blue dye (Sigma-Aldrich, St. Louis, Missouri, United States). Viable cells were counted using a hemocytometer.

**Lucifer yellow penetration assay.** HPV-KER cells were seeded on porous 12-well plates (pore size 0.4 µm, Corning, New York, USA) at a density of $1.5 \times 10^5$ cells/well using KSFM (Life Technologies, Carlsbad, USA). Ca-high cultures were established and treated with *C. acnes* 889 strain (MOI = 300) for 24 and 72 h. Lucifer yellow (LY) penetration experiments were carried out in HBSS containing 100 µM LY (Sigma-Aldrich, Saint Louis, Missouri, USA). HBSS-LY media was added to the upper chamber and samples were collected from the bottom chamber after 30 min of incubation using standard culturing conditions (5% v/v $CO_2$, 37 °C). Fluorescence intensities were measured with a BMG FLUOstar OPTIMA Fluorescence plate reader (Gemini BV Laboratory, Netherland) and relative fluorescence intensities were calculated. Fluorescence intensities were normalized to the time-matched untreated control values.

In the case of the OS models, *C. acnes* 889 strain (15 µl, $10^9$ CFU/sample) was pipetted onto the upper, epidermal part of the skin samples, which were then incubated for 72 h. For the transport experiments, LY diluted to 1 mM LY with PBS (15 µl/sample) was applied to the top. After 3 h, skin samples were collected using 6 mm punch biopsies and embedded in Shandon Cryomatrix (Thermo Fischer Scientific, Massachusetts, USA) for frozen sectioning. Sections (6 µm) were cut from the samples, and LY dye penetration was analysed using a Zeiss Axio Imager Z1 fluorescence microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with an AxioCam MRm camera.

**Antibiotic treatment of C. acnes co-cultured HPV-KER monolayers.** The effect of antibiotic treatment of *C. acnes* co-cultured HPV-KER monolayers was observed in real time using the RTCA system. HPV-KER cells were plated at a density of 30,000 cells/well in fibronectin coated 96-well E-plates. After the cultures reached confluency, they were treated with *C. acnes 889* strain (MOI: 100) and, 44 h later, 1% or 5% AB/AM was added to the cultures. Each treatment was performed in five technical replicates. Impedance values were measured every 60 min for 120 h, and a dimension-free cell index (Ci) was calculated from the data. Ci (average of the technical replicates) tracings were normalized to values recorded at the addition of the bacterium to the cultures, and the resulting nCi values were plotted.

**Statistical analysis.** Unless otherwise noted, all the data are presented as mean ± SEM for three independent experiments. For xCELLigence analysis, treatment was performed in at least five times. For real-time RT-PCR and trypan blue exclusion experiments, each treatment was performed at least three times. For western blot analysis and IHC staining, each condition was repeated once in each independent experiment. Data were compared using paired Student's t-test and one-way ANOVA with post-hoc Tukey-test. A probability value of less than 0.05 was considered significant.

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## Author contributions

B.Sz.B. and K.Sz. designed the experiments. B.Sz.B., L.E. performed the experiments. E.U. and K.B. provided the *C. acnes* strains used in our experiments. B.Sz.B., L.K. and K.Sz. performed data analysis and wrote the manuscript. L.K. and K.Sz. were involved in the coordination of the study.

## Competing interests

The authors declare no competing interests.

## Additional information

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# II.

# A bőrünkön élő mikróbák szerepe az egészséges bőrben és az acne vulgaris kialakulása során

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**Testünk különböző felszínein számos baktérium, gomba és vírus időszakosan vagy állandóan együtt él velünk, bonyolult kapcsolatot alakít ki sejtjeinkkel. Bőrünk sejtjei és az itt élő mikrobák komplex ökoszisztémát alakítanak ki. A kután mikrobiom tagjai elengedhetetlenek a bőr egészséges működéséhez. Az egyensúlyi állapot felborulásával egyébként ártalmatlan mikrobák is patogénként viselkedhetnek. Ez játszódhat le az acne vulgaris patogenezise során, ahol a *Propionibacterium acnes* baktérium szerepét régóta feltételezzük. Tanulmányunkban a kután mikrobiom legfontosabb sajátságait, valamint az akné kialakulásában szerepet játszó folyamatokat mutatjuk be.**

A testünket alkotó számos különféle sejttípus nem mindegyike humán eredetű. Ennek igazolása egy német születésű gyermekorvos, Theodor Escherich nevéhez fűződik, aki munkája során egészséges és beteg gyerekek székletmintájából izolálta a később az ő tiszteletére *Escherichia coli*-nak elnevezett baktériumot. Kortársa, Louis Pasteur már azt is feltételezte, hogy a normál humán flórára az élethez is szükség van.[1]

A szervezetünk külső, első védelmi vonalát alkotó bőr egyik legfontosabb feladata a környezetünkben található veszélyes anyagok és mikrobák (baktériumok, gombák, vírusok) kívül tartása annak érdekében, hogy elkerüljük a különféle betegségekhez vezető fertőzéseket. Az evolúció során kültakarónk sajátságai oly módon alkalmazkodtak e funkció ellátásához, hogy megnehezítsék az esetleges támadók szervezetbe jutását. Ennek ellenére vannak olyan mikroorganizmusok, amelyek bőrünket választották élettérül, és átmenetileg vagy állandóan jelen vannak a testünk feszínén. A legújabb kutatási eredmények pedig azt is igazolják, hogy ezek a mikrobiális fajok nem egyszerű passzív szemlélők, hanem a különböző humán sejtekkel együtt komplex ökoszisztémát alakítanak ki.[2]

## A HUMÁN MIKROBIOM PROJEKT (HMP)

Pasteur elképzelése, vagyis hogy a testünk különböző részein élő mikrobák fontos és nélkülözhetetlen részét képezik szervezetünknek, egy évtizede kapott különös figyelmet. Ekkortájt indultak útjukra azok a vizsgálatok, amelyek célja a különböző szerveket benépesítő mikrobiális közösségek pontos összetételének, feladatának és sajátságainak

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meghatározása volt. Eleinte egymástól független kutatások folytak ebben a témában a világ különböző laboratóriumaiban, hamar kiderült azonban, hogy hasznos lenne, ha széles körű nemzetközi összefogás szerveződne. Szükség volt olyan standardizált módszerekre, amelyek segítségével az egyes munkacsoportok által kapott eredmények összevethetők. Emellett a nagy nemzetközi konzorcium kialakításával lehetővé vált nagyobb léptékű pályázati források elnyerése is.

E megfontolások alapján 2008-ban indult útnak a Humán Mikrobiom Projekt (HMP). A program a posztgenomi éra legnagyobb, szekvenálásos eljárásokon alapuló munkájává vált, melynek célja a különböző testtájékokon élő mikrobiális közösségek összetételének meghatározása, biológiai szerepük karakterizálása lett.[3] A HMP tevékenységének eredményeképpen az elmúlt néhány évben már sok érdekes adat gyűlt össze a nemzetközileg koordinált kutatásoknak köszönhetően.

## A MIKROBIÓTA, A BŐR MIKROFLÓRÁJA

Testünk különböző felszínein az állandó hőmérséklet és páratartalom, a folyamatosan rendelkezésre álló tápanyagok lehetővé teszik egyes baktérium- és gombafajok, valamint vírusok megtelepedését. A bőrnek, a bélcsatornának és az urogenitális traktus egyes részeinek kolonizáltsága már régóta ismeretes, azonban újabb eredmények alapján korábban sterilnek gondolt szervek, pl. a tüdő és a placenta is saját mikrobiális közösséggel rendelkezik.[4,5]

A humán sejtek és a helyi mikróbaközösségek kapcsolata nagyon bonyolult. Korábban azt gondoltuk, hogy a velünk élő baktériumok, vírusok és gombák úgy használják az egyes élőhelyeken található erőforrásokat, hogy közben nem okoznak változást szervezetünk működésében. Az ilyen életközösséget az ökológusok kommenzalistának nevezik. Az elmúlt évek kutatási eredményei alapján azonban ma már úgy tűnik, hogy ezek a fajok és metabolikus termékeik fontos szerepet játszanak a gazdaszervezet különféle biológiai funkcióinak ellátásában. Szabályozhatják a kolonizált humán szervek fejlődését, differenciációs folyamataikat, és segíthetnek fenntartani megfelelő működésüket.[6]

A bőr mikroflórája az epidermisz felszínén és a piloszebáceus egységek területén él. Ebben a közösségben eddig mintegy ezer különböző bakteriális fajt, gombát (dermatofitonok) és vírust azonosítottak, melyek 19 különböző törzsbe tartoznak. Ezek közül a négy leggyakoribb baktériumtörzs az Actinobacteria (*Propionibacterium*- és *Corynebacterium*-fajok), a Proteobacteria, a Firmicutes (*Staphylococcus*-fajok) és a Bacteroidetes.[7]

Bőrünk faj szintű mikrobiális összetételét különböző faktorok (egyéni sajátságok, életstílus, környezet) befolyásolják, és az életkörülmények megváltozása a közösséget alkotó fajok összetételének gyors megváltozásához járul hozzá.

## KORAI KOLONIZÁCIÓ

A bőr kolonizációja a születéskor kezdődik, és kezdeti összetételét a születés módja (természetes szülés vagy császármetszés), helye (kórházi vagy otthoni szülés) és körülményei (pl. antibiotikumok használata) határozza meg.[8] Természetes szülés során a csecsemők az anya vaginális mikrobáival kerülnek kontaktusba, így *Lactobacillus-*, *Prevotella-*, *Atopobium*- és *Snethia*- fajok is jelen vannak. Ezzel szemben a császármetszéssel született gyerekek a környezetből szerzik be az első mikrobákat (főként *Staphylococcus*-fajokat és más, bőrről származó baktériumokat), melyek nem feltétlenül az édesanyjuktól származnak. Ez azért is nagyon fontos, hiszen az újszülöttek különböző testtájainak (pl. bélcsatorna, szájüreg, bőr) kolonizációja a bőr flórájából indul, így ezeknek a közösségeknek az összetétele kezdetben megegyezik. A császármetszéssel született babák bélcsatornájában a táplálékuk feldolgozásához szükséges *Lactobacillus-*, *Bifidobacterium*- és *Bacterioidetes*-fajok kolonizációja késhet, ami hozzájárulhat a napjainkban gyakran megfigyelhető korai táplálkozási problémák kialakulásához.[9]

A korai kolonizáció emellett azért is kritikus esemény, mert mintázatának megváltozása kedvezőtlenül befolyásolhatja az immunrendszer fejlődését. A csecsemők esetében a környezeti mikrobákkal való első találkozások kritikusak, így ha ezek ideje és mintázata nem megfelelő, az a későbbiekben különféle atópiás, krónikus gyulladásos és allergiás betegségek kialakulásához vezethet.[10]

Annak érdekében, hogy a császármetszéssel született csecsemők is a megfelelő mikrobákkal kerüljenek kapcsolatba életük első óráiban, az elmúlt években transzplantációs kísérletek indultak, melyek során az anya vaginális flórájából származó baktériumokat juttatnak az újszülöttek bőrére. A kezdeti, egyelőre kisszámú egyén bevonásával végzett vizsgálatok eredményei ígéretesek, az elkövetkező években pedig érdekes lesz annak tanulmányozása, hogy az eljárásnak milyen hosszú távú következményei lesznek a növekvő gyermekek egészségi állapotára.[11]

A bőr korai kolonizációját követően a mikroflóra összetétele 1 és 2 éves kor között stabilizálódik. Legfontosabb alkotóelemei ekkor a Streptococcaceae család és más Firmicutes-, Bacterioidetes-, β- és γ Proteobacteria-törzsek tagjai. A közösség összetétele ezt követően csak a pubertás idején változik jelentősebben, amikor az endokrin változások hatására follikuláris hiperplázia, következésképpen megnövekedett faggyúszekréció figyelhető meg.[12] A mikrobiális közösségek összetételének elemzésével a kutatók megállapították, hogy a szexuális éréssel párhuzamosan a mikroflóra összetétele is átalakul. A faji diverzitás észrevehetően csökken, és a Corynebacteriaceae, valamint a Propionibacteriaceae család tagjai előnybe kerülnek. Ezeknek a lipofil mikrobáknak

a mennyisége jelentősen megnövekszik, míg más fajok mennyisége a megváltozott környezeti feltételek és a lipofilekkel való kompetíció eredményeképpen nagymértékű csökkenést mutat.[13]

## A PUBERTÁS UTÁNI MIKROFLÓRA JELLEGZETESSÉGEI

A serdülőkori átmeneti időszak után a kután mikroflóra összetétele újra stabilizálódik. Érdekes azonban az a megfigyelés, hogy a fajösszetétel nem homogén a teljes testfelületen, ugyanis a finom anatómiai és fizikokémiai sajátságok az egyes testtájakon jelentős eltéréseket mutatnak. Nedves, száraz vagy zsíros sajátságokat mutató területeket különböztethetünk meg. Griece és munkatársai szerint a faggyúban gazdag területeket Actinobacteria- és Firmicutes-, a nyirkos területeket (pl. hónalj, ujjak közötti területek, lágyékhajlat) főként Corynebacteria-fajok kolonizálják. Érdekes módon a száraz területek fajgazdagsága a legnagyobb, pl. a felkar és a farpofák területén az Actinobacteria, Proteobacteria, Firmicutes és Bacteriodetes kevert populációja figyelhető meg.[7]

A kután mikroflóra összetételében eltérés van a nők és a férfiak között. Ennek hátterében egyrészt a bőr finom anatómiai és fiziológiai tulajdonságainak (vastagság, pH, szébum összetétele és mennyisége, kozmetikumok használata) különbségei állnak. Reproduktív szerveink is speciális mikrobiommal rendelkeznek, és az ezek területén megtalálható fajok a környező anatómiai régiókra is átterjedhetnek. A nők esetében gyakran találunk *Lactobacillus*- és *Gardnerella*-, míg férfiaknál *Corynebacterium*-fajokat pl. a farpofák területén.[14]

## A MIKROBIOM AZ EGÉSZSÉGES BŐRBEN ÉS PATOGÉN FOLYAMATOKBAN

A kiegyensúlyozott kölcsönhatás a velünk élő mikrobák és a bőrsejtek között fontos nemcsak a bőr, de az egészséges szervezet működésének kialakításában és fenntartásában is.[15] A különböző baktériumok és gombák olyan anyagokat termelnek, melyeket szervezetünk sejtjei tápanyagként hasznosíthatnak és amelyek védelmet biztosíthatnak egyes patogén mikrobák ellen. Előnyösen befolyásolhatják az immunrendszerünk működését, és elősegíthetik egyes szövetek differenciációját, megújulását. Nem csoda, hogy ennek a finom és bonyolult egyensúlynak a felborulásával diszbiózis alakulhat ki, melynek során szervezetünk sebezhetővé válik.

Ebben az instabil állapotban már az egyébként ártalmatlan, a mikrobiomhoz tartozó fajok is kórokozóvá válhatnak, illetve lehetővé válhat patogén fajok megtelepedése is, ami végső soron különféle betegségek létrejöttéhez vezethet. Ilyen betegség pl. a szeborreás dermatitisz, melynek kialakulásában *Malassezia*-fajok, az atópiás dermatitisz, ahol a *Staphylococcus epidermidis,* vagy az acne vulgaris, melyben a *Propionibacterium acnes* baktérium játszik fontos szerepet.[16]

## AZ ACNE VULGARIS PATOGENEZISE

A pubertás korban bekövetkező hormonális változások, a kialakuló androgéntúlsúly, faggyúmirigy-hiperplázia és megnövekedett faggyútermelés kedvező körülményeket teremt lipofil baktériumok növekedése számára. Ekkor változik a „gyermekkori" mikrobiom, a Streptococcaceae, Firmicutes β- és γ-Proteobacteria dominanciáját felváltja az inkább a felnőttkori mikrobiomra jellemző összetétel, ahol a Corynebacteriaceae és a Propionibacteriaceae családokba tartozó fajok kerülnek túlsúlyba.[12] A változások hajtóereje a bőr és a mikrokörnyezet sajátságainak megváltozása, és az átmenti időszakban, a felnőttkorra jellemző ökoszisztéma stabilizálódása előtt instabil állapot, ún. diszbiózis alakulhat ki.

A folyamatok egyik főszereplője, a *P. acnes* baktérium Gram-pozitív, anaerob, fermentáló, lipofil, pálca alakú mikroba, mely a bőr felső rétegeiben és a piloszebáceus egységek (pilosebaceous unit, PSU) területén él. Számos olyan enzimet és anyagcsereterméket szekretál, amely hatással bír más mikrobákra. Ilyen enzimek többek között a lipázok, melyek segítségével a baktérium a szébum lipidjeiből szabad zsírsavakat állít elő. Emellett rövid szénláncú zsírsavakat is szekretál, köztük ecetsavat, propionsavat és vajsavat, mely molekulák mindegyike szintén rendelkezik antibakteriális hatással.[17] Emellett a fenti anyagok savas tulajdonságaik révén hozzájárulhatnak a bőr jellegzetes, savas pH-jának a fenntartásához is, amely korlátozza számos mikroba megtelepedését és növekedését. Mindezek alapján nem meglepő, hogy a pubertáskori változások idején a *P. acnes* gyorsan dominánssá válik, különösen az olyan, faggyúban gazdag területeken, mint az arc, a mellkas és a hát felső része.

Korábbi kutatásokból tudjuk, hogy a *P. acnes* baktérium képes a különféle bőrsejtek felszínén található patogénfelismerő receptorok, pl. a Toll-like receptorok aktiválására és az érintett sejtekben jellegzetes jelátviteli folyamatok elindítására. Ennek eredményeképpen veleszületett immun- és gyulladásos folyamatok is aktiválódnak, és az ezekben a folyamatokban fontos szerepet játszó mediátorok – gyulladásos citokinek, kemokinek, antimikrobiális peptidek – kifejeződése fokozódik. Saját eredményeink alapján az is megállapítható, hogy ezek a folyamatok dózisfüggőek, a bekövetkező változások mértéke nagymértékben függ a bőrsejtek környezetében található baktériumok mennyiségétől. A szőrtüszőkben található sejtek (keratinociták, szebociták) megváltozott működése és a kialakuló gyulladásos folyamatok súlyossága függvényében jellegzetes bőrtünetek alakulhatnak ki: komedók, papulák és pusztulák, illetve súlyosabb esetekben nódusok és ciszták.[15]

A fenti események hátterében zajló sejtes és molekuláris eseményekről sok adattal rendelkezünk, de a részleteiket a mai napig nem sikerült teljes egészében feltárni. Régóta ismerjük a *P. acnes* hatására a keratinocitákban induló TLR szignálfolyamatok jelentőségét az immun- és gyulladásos események kialakításában, azonban csak az elmúlt években sikerült megmutatnunk, hogy a gyulladásos folyamatokat elősegítő faktorok mellett a baktérium hatására antiinflammatórikus molekulák is termelődnek. Ezeknek nagyon fontos szerepük lehet abban, hogy bár a sejtjeink érzékelik a környezetükben élő baktériumok jelenlétét az egészséges bőrben, egyensúlyi helyzetben nem történik észlelhető immunaktiváció. Ezek a molekulák tehát fontos feladatot láthatnak el a bőr homeosztatikus viszonyainak fenntartásában (Erdei Lilla, közlés alatt).

A keratinociták mellett a follikuluszokat alkotó másik sejttípus, a szebociták szerepe napjainkban is intenzív kutatás tárgyát képezi. Korábban úgy gondoltuk, hogy feladatuk kimerül a bőr lipid barrierjének kialakításában fontos szerepet játszó faggyú termelésében. A szébum mennyisége és összetétele azonban nem állandó, mindkét paraméter tekintetében eltérést figyelhetünk meg egészséges egyének és aknés betegek mintáit megvizsgálva. Ma már egyre több adat utal arra is, hogy a szebociták maguk is jelentős szerepet játszanak a különféle mediátorok termelésében, és ezek a molekulák a környezetben található lipidekkel együttműködve aktív szerepet játszanak a gyulladásos környezet létrehozásában, valamint a különböző immunsejtek (dendritikus sejtek, makrofágok) aktivációjában.[18]

Bár az akné tünetei a bőrre lokalizálódnak, a helyben induló patogén folyamatokról végső soron az adaptív immunrendszer sejtjei is tudomást szereznek. A bőrben lokálisan termelődő kemotaktikus faktorok (pl. IL-8 interleukin) hatására a kialakuló léziók környezetébe monociták, makrofágok és T-sejtek vándorolnak. Végső soron $T_{h1}$ és $T_{h17}$ irányú T-sejt-aktivációs folyamatok indulnak, melyek létrejöttében a follikuluszban található különböző sejtek – a keratinociták, a szebociták, a dendritikus sejtek és a *P. acnes* – interakciója fontos szerepet játszik. A legújabb kutatási eredmények a felsorolt sejtek eltérő reakciókészségét sejtetik. A baktérium hatására az aktiválódó dendritikus sejtek döntően $T_{h1}$ irányú T-sejt-differenciációt indítanak. Ezek a folyamatok azonban szebociták által termelt mediátorokat tartalmazó felülúszó jelenlétében a $T_{h17}$ irányába tolódhatnak el, ami felveti annak lehetőségét, hogy ezek a sejtek fontos szerepet játszanak a follikuluszok homeosztatikus viszonyainak fenntartásában.[19] Nem meglepő ezek alapján az sem, hogy a faggyúmirigyekben gazdag területek speciális tolerogén jellegű sajátságokkal rendelkeznek. Ez a tulajdonság nagyban hozzájárulhat ahhoz, hogy a bőr felszínén és a PSU területén található mikrobák nem okoznak immunaktivációt az egészséges bőrben.[20]

Összességében, az acne vulgaris kialakulása során a veleszületett immunrendszer aktiválódása mellett adaptív immunfolyamatok is bekapcsolódnak a patogén események szabályozásába. Ez lehetővé teheti egy magasabb szintű, összetettebb kontrollmechanizmus kialakulását, mely végső soron, a serdülőkort követően már képes a bőr sejtjei és a mikrobiom közötti egyensúly fenntartására. Ez lehet az oka annak is, hogy a tinédzserkori átmeneti időszakot követően a bőrtünetek az érintett egyének nagy hányadánál maguktól, nyom nélkül elmúlnak a felnőttkor kezdetére.

## MIÉRT LÁTSZIK CIVILIZÁCIÓS BETEGSÉGNEK AZ AKNÉ?

Korábbi vizsgálatok eredményei alapján úgy tűnik, hogy az akné a fejlett, nyugati típusú életmódot folytató társadalmak betegsége. Irodalmi adatok arra utalnak, hogy az eddig vizsgált elszigetelt, természetközeli életmódot folytató közösségekben (pl. eszkimók, bantu négerek, Okinava szigetének lakói a II. világháborút megelőzően) ez a bőrbetegség gyakorlatilag ismeretlen. Ez azért is érdekes megfigyelés, hiszen napjainkban a nyugati társadalmakban élő serdülő populáció 80–90%-át érinti kisebb-nagyobb mértékben a kórkép.[21]

Mi lehet a fenti különbségek oka? Jelenleg úgy gondoljuk, hogy a velünk élő mikróbák adhatnak választ erre az érdekes kérdésre. A HMP keretében az Amazonas dzsungelében élő természeti nép (janomami) vizsgálata során a kutatók azt találták, hogy e populáció felnőtt tagjainak kután mikroflórája sokkal komplexebb és kiegyensúlyozottabb. Esetükben a *P. acnes* dominanciája egyáltalán nem jellemző, szemben azzal, amit a nyugati típusú életmódot folytató egyéneken megfigyelhetünk.[22] Sajnos arról egyelőre nincs adatunk, hogy a janomami populáció serdülő tagjai szenvednek-e aknés tünetektől, azonban a fentebb említett tanulmányok arra engednek következtetni, hogy más természeti népekhez hasonlóan a betegség valószínűleg itt sem jelentkezik.

A fentiek alapján ok okozati összefüggést feltételezünk a *P. acnes* túlsúlyának kialakulása és az akné patogenezise között. De mi okozhatja ennek a lipofil baktériumnak a dominanciáját a posztpubertáns bőrmintákban? Úgy gondoljuk, hogy az emberiség történelme során életmódunk drámai változása miatt egyes mikrobák eltűntek a mikroflóránkból a higiénés viszonyok átalakulása miatt. Ez az „eltűnő mikrobióta" (disappearing microbiota) elmélet Martin Blaser tollából származik.[23] Ennek alapján különösen az ipari forradalmat követően elveszhettek a kután mikrobióta egyes tagjai, mégpedig azok, amelyek a korábbiakban képesek voltak a *P. acnes* baktérium kordában tartására mikrobiális kompetíció révén. Ezek hiányában a serdülőkori fokozott faggyútermelés hatására a baktérium olyan növekedési előnyt szerez, ami egy

átmeneti időszakra diszbiózis kialakulásával járhat együtt. Mivel a fenti változások evolúciós léptékben számolva új keletűek, szervezetünknek még nem volt ideje megfelelően alkalmazkodni a megváltozott körülményekhez. Egy néhány éves átmeneti időszakot követően azonban immunrendszerünk képessé válik a patogén folyamatok kontrollálására, annak ellenére, hogy a *P. acnes* a későbbiekben is fontos eleme a kután mikrobiótának.[16]

## ÖSSZEGZÉS

A testünk és a velünk élő mikróbák közötti komplex kölcsönhatás elengedhetetlen nem pusztán a bőr, de a teljes szervezet egészséges működésének fenntartásában is. Ha ez az egyensúly felborul, a kialakuló diszbiózis miatt már a mikroflóra részét képező, „ártalmatlan" baktériumok is patogénné válhatnak. Ez történhet a *P. acnes* baktériummal is a serdülőkor idején, az acne vulgaris kialakulásakor. Az érintett egyének jelentős részénél a betegség magától elmúlik a felnőttkor kezdetére, ez immunrendszerünk egyes elemeinek, azok komplex és összehangolt működésének köszönhető.

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