PROF. BRIGITTE DRENO (Orcid ID : 0000-0001-5574-5825)

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Cutibacterium acnes phylotypes diversity loss: a trigger for skin inflammatory process

Cutibacterium acnes phylotypes diversity loss can trigger skin inflammation

Marie-Ange Dagnelie[1], Stéphane Corvec[2], Mélanie Saint-Jean[1], Jean-Michel Nguyen[3], Amir Khammari[1], and Brigitte Dréno[1*]

[1] Dermatology department, CHU Nantes, CIC 1413, CRCINA, University Nantes, Nantes France
[2] Bacteriology department, CHU Nantes, CRCINA, University Nantes, Nantes France
[3] Biostatistical department, CHU Nantes, CIC 1413, CRCINA, University Nantes, Nantes France

Corresponding author
Pr Brigitte Dréno
Department of Dermatology
Nantes University Hospital
1 Place Alexis Ricordeau
44035 Nantes Cedex 01
France
Phone: 00 33 240083118
Fax: 00 33 240083117
E-mail: brigitte.dreno@wanadoo.fr

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Conflict of interest

The authors S. Corvec, M. Saint-Jean and JM Nguyen have no conflict of interest to declare. The authors B. Dréno (director of the study), A. Khammari (co-director of the study) and MA Dagnelie (Ph.D. student) declare a potential conflict of interest as the study was funded by Galderma R&D - CUTIS (Sophia Antipolis, France).

Abstract

Background

Acne has long been understood as a multifactorial chronic inflammatory disease of the pilosebaceous follicle, where *Cutibacterium acnes* (subdivided into six main phylotypes) is a crucial actor. In parallel, the loss of microbial diversity among the skin commensal communities has recently been shown as often accompanied with inflammatory skin disorders.

Objective

This study investigated the association of *C. acnes* phylotype diversity loss and the impact on Innate Immune System (IIS) activation.

Methods

The IIS response of skin after incubation with phylotypes IA1, II or III individually and with the combination of IA1+II+III phylotypes, was studied in an *in vitro* skin explant system. The inflammatory response was monitored by immunohistochemistry and ELISA assays, targeting a selection of Innate Immune Markers (IIMs) (IL-6, IL-8, IL-10, IL-17, TGF-β).

Results

IIMs were significantly upregulated in skin when being incubated with phylotype IA1 alone compared with the combination IA1+II+III. In parallel, ELISA assays confirmed these results in supernatants for IL-17, IL-8, and IL-10.

Conclusion

We identify the loss of *C. acnes* phylotype diversity as a trigger for IIS activation, leading to cutaneous inflammation. These innovative data underline the possibility to set up new approaches to treat acne. Indeed, maintaining the balance between the different phylotypes of *C. acnes* may be an interesting target for the development of drugs.

Introduction

Acne has long been understood as a complex multifactorial inflammatory disease. Currently, three key factors are described as being implicated in acne development: hormonally-stimulated sebum production, abnormal keratinization of the pilosebaceous duct, and skin colonization by *Cutibacterium acnes*, formerly *Propionibacterium acnes* (1). Over the last five years, the Innate Immune System (IIS) started to be highlighted as the fourth key player and microbiota as the fifth key player in acne physiopathology (2–5).

Cutibacterium acnes (*C. acnes*) is a Gram-positive bacterium, growing in anaerobiosis. It is usually found as living in pilosebaceous units, and *C. acnes* mainly uses lipids as a source of energy, and its metabolism produces propionic acid (6). This bacterium has been described both as a commensal of the human skin playing a role in the balance of normal skin microbiota (7), and as a pathogen bacterium (8). For instance, *C. acnes* implication was previously reported in cutaneous dermatoses such as acne and folliculitis (3,9), but also in other types of affections including deep medical device infections, prostate cancer, and sarcoidosis (10–12). *Cutibacterium acnes* strains have long been classified into several subtypes, currently, it is subdivided into six main phylotypes : IA1, IA2, IB, IC, II and III (13,14).

Recent studies on acne have begun to determine how far these four factors interact, and the role of the IIS is nowadays better appreciated. On one hand, inflammation has been demonstrated in all steps of acne lesions: as soon as the microcomedo, then comedo, inflammatory lesion, and even 'post-inflammatory' erythema (hyperpigmentation), and scars (15). *C. acnes* has a key role in IIS activation and cutaneous inflammation process, as it is able to induce pro-inflammatory cytokines releasing by keratinocytes and immune cells (including macrophages and monocytes): IL-8 (16), IL-17 (17) and IL-1β (18). It is also known to induce the expression of TLRs, mainly TLR-2 (19). In parallel, increasing insights into skin microbiota shed the light on an interesting theory, in several cutaneous illnesses as atopic dermatitis, psoriasis and vitiligo: the loss of microbial diversity among the skin commensal communities (dysbiosis) would be associated with cutaneous inflammatory diseases (20–22). Concerning *acne vulgaris*, some recent papers suggest that specific *C. acnes* subtypes could play a key role in the severity of inflammatory acne (23–25). Moreover, we reported a clear loss of *C. acnes* phylotype diversity in severe back acne context (26). Consequently, it appears relevant to elucidate whether not only *C. acnes* phylotype, but also the loss of diversity can modulate IIS activation and thus, could play a role in skin inflammation severity, in acne context. In this original article, we propose an *in vitro* system to clarify this crucial question.

We co-cultured healthy skin explants with different clinical *C. acnes* strains previously well-characterized. We evaluated explants IIS activation level, comparing the impact of *C. acnes* phylotype alone, with a mixture of several phylotypes mimicking the diversity found in healthy context. IIS activation evaluation was performed by ImmunoHistoChemistry (IHC), and ELISA assays.

Materials and Methods

Non-lesional skin origin from acne patients

At the first visit, acne patients were selected according to inclusion criteria: age (16-35 years-old), at least 2 nodules on the back, respect of wash-out periods for acne drugs including systemic antibiotics (1 month), oral retinoids (6 months) and topical treatments (2 weeks). All patients signed informed consent and this translational monocentric study was approved by the French health authorities (ANSM) under number 151141B-42 and ethic committee (CPP) under number 21-15. From these patients, biopsies were performed taking a non-lesional skin, a nodule and a papule.

Healthy skin origin and explants

Healthy skin (abdominoplastie) was kindly provided by the Surgery Department at Nantes University Hospital, as previously validated (25). It is obtained from healthy skin of abdominoplasties as skin biopsies of the face/back of healthy individuals were considered as not ethical. As previously reported (27), this biological system has some limits, but it has been widely used previously (25,28), and it enables to take into account three main layers of cells including dermis (mainly fibroblasts), epidermis (keratinocytes) and *stratum corneum* (corneocytes) ; and also immune effectors that make the normal human skin.

Cutibacterium acnes clinical strains and characterization

Selected bacterial isolates were obtained from the bacteriological collection, stored in the Bacteriology Unit at Nantes University Hospital facilities. All strains used were previously characterized accurately according to three levels of characterization previously described: phylotype, clonal complex and SLST-type (13,14,23). A summary of the clinical strains used and their characterization is presented in table 1.

Bacteria lysates

To ensure bacteriological safety of cell culture incubator and to avoid an excess of bacteria multiplication during the assay, bacteria in lysate forms were preferred to viable bacteria inoculum. Immune and keratinocytes/sebocytes stimulations using bacteria in lysate forms were previously widely described, and based on these publications, we were able to use lysates to perform the experiments (25,29–31). To create bacteria lysates, all bacterial suspensions were calibrated at a DO=1.00±0.15, heat-treated, and entered into a series of freezing/thawing and freezing/sonication cycles to ensure efficient mechanical lysis of their Gram-positive cell walls. Viability controls were systematically performed before and after the lysis process to ensure that no viable bacteria were recovered before cocultures experiments were started. Protein quantifications were then performed using BCA assay (Thermo Fischer Scientific, cat. #23227) and lysates were used at a 5 µg/mL final concentration.

Cocultures experiments

Skin explants were cocultured with *C. acnes* lysates for 6 h, at 5 µg/mL protein final concentration, in DMEM high glucose GlutaMAX™ Supplement, pyruvate (ThermoFisher Scientific, cat.# 10569010), as previously described (25). Explants were cocultured either with a *C. acnes* phylotype alone (IA1, II or III), or with a combination of phylotypes in order to mimic healthy phylotypes diversity *in vitro* (IA1+II+III). Positive control was performed by incubating explants with LPS from *Escherichia coli* (Sigma Aldrich, L4391-1MG) at a 5 µg/mL final concentration. Negative control condition was performed by incubating explants with culture media only.

ELISA assays

ELISA assays were performed according to the manufacturer's instructions, for following cytokines: IL-1β, IL-6, IL-8 (also called CXCL-8), IL-10, IL-17 and TGF-β (Affymetrix, IL-1β cat. #88-7261-22, IL-6 cat. #88-7066-22, IL-8 cat. #88-8086-22, IL-10 cat. #88-7106-22, IL-17 cat. #88-7176-22, and TGF-β cat. #88-8350-22).

Immunohistochemistry

Immunohistochemistries were performed on Non-Lesional Skin (NLS) from patients with severe acne of the back, and on NLS from healthy individuals, targeting IIMs (IL-1β, IL-17, IL-10, β-defensin-2, TGF-β, and TLR-2), as previously described (25). The same markers were also investigated in explants after 6h incubation with the different bacterial lysates combinations. The used antibodies are summarized in the table 2. The slides were double-blinded read by two independent readers. The averages of both scores were taken into account for the analysis of the results.

Statistical analysis

First, innate immune markers studied in non-lesional skins (NLS) from acne (n=12) and healthy were compared (n=13). The markers were assessed by two independent readers and the mean of their measures was used for statistical analyses. Comparisons between healthy and acne skins were performed using General linear Models (GLM) and were confirmed using a non-parametric test (Wilcoxon unpaired test). Secondly, the experimental plan analyzed was made of randomized block of 9 conditions repeated within 3 healthy skins. The markers were assessed by two independent readers and the mean of their measures was used for statistical analyses. General linear Models were used to limit the number of t-tests and to focus the comparisons between the control block and the others experimental situations. For

all these analyses, R V3.4 software was used (R Foundation for Statistical Computing, Vienna, Austria).

Results

The innate immune system is abnormally activated in acne patients

Firstly, we compared the basal level of cutaneous innate immune system activation in normal skin of healthy subjects with normal skin of patients with nodular acne on the back. Four out of six tested IIMs were significantly upregulated (1.2 to 1.8 fold) in NLS from acne group comparing with NLS from healthy group (Fig. 1).

TLR-2 and β-defensin-2 were respectively nearly 1.8 and 1.7-fold more expressed in NLS from acne group than NLS from healthy individuals (respectively $p<0.001$ and $p<0.001$) (Fig. 1). In addition, inflammation modulators such as TGF-β and IL-10 were respectively 1.5 and 1.2-fold more expressed in NLS from acne group than NLS from healthy individuals, but with no significant differences (TGF-β, $p=0.425$ and IL-10, $p=0.25$). In contrast, IL-1β was found to be less expressed in NLS from acne patients than healthy individuals (IL-1β, $p<0.001$). There was no significant difference for IL-17 expression between healthy and acne groups (IL-17, $p=0.34$). Taken together, these data show that IIS in NLS is already up-activated in acne patients, particularly *via* TLR-2 and β-defensin-2 overexpressions.

Phylotypes IA1, II and III individually induced a significant IIS activation comparing with negative control

First, IIS activation was evaluated by immunohistochemistry targeting IIMs on explants incubated with IA1, II or III phylotypes individually, comparing with negative control. First of all, IHC showed a significant overexpression of TLR-2 (p=0.00054), IL-1β (p=0.0223), IL-10 (p=0.00153), IL-17 (p=0.00048), and β-defensin-2 (p=0.00011) in explants incubated with phylotype IA1, in comparison with negative control (Figs 2-3). TGF-β was the only marker with no significant variation between the conditions. In addition, IHC showed a significant increase of IL-1β (p=0.0176), IL-10 (p=0.01578), TLR-2 (p=0.00803), and β-defensin-2 (p=0.00390) in explants incubated with phylotype III comparing with negative control (Fig. 2). Finally, IHC showed a significant increase of IL-10 (p=0.01578), TLR-2 (p=0.00075), β-defensin-2 (p=0.00261) in explants incubated with phylotype II comparing with negative control (Fig. 2).

Restoration of phylotype diversity decreases the inflammatory response of healthy skin explants in vitro

Our hypothesis is that phylotype diversity (rather associated with healthy skin condition) would be able to induce a lower inflammatory response comparing with phylotypes only (IA1, II and III separately). To elucidate this question, we evaluated the IIMs expression level of skin explants incubated with either *C. acnes* IA1, II or III phylotype singly or with a combination of IA1+II+III phylotypes. We observed a significant increase of markers IL-1β (p=0.0374), IL-10 (p=0.00630), IL-17 (p=0.00290), TLR-2 (p=0.00406), and β-defensin-2 (0.00290), in explants incubated with phylotype IA1 alone, than with the combination IA1+II+III (Figs 2-3). The only marker which did not show any significant variation was TGF-β (Fig. 2). For example, β-defensin-2 was 4-fold less expressed (p=0.00290) in explants

incubated with the mixture of phylotypes IA1+II+III compared to IA1 phylotype used alone (Figs 2-3). In the same manner, IL-1β was 3-fold less expressed ($p=0.0297$) in explants incubated with the mixture of phylotypes IA1+II+III (Fig. 2). In parallel, there was a significant increase in IL-1β ($p=0.0297$), in explants incubated with phylotype III only, in comparison with the combination (IA1+II+III) (Fig. 2). Also, there was a significant increase in TLR-2 ($p=0.00572$), in explants incubated with phylotype II only, in comparison with the combination (IA1+II+III) (Figs 2-3).

In summary, IIMs were upregulated in skin when being incubated with only one phylotype (IA1, II or III) comparing with a mixture of phylotypes (IA1+II+III). Explants incubated with the combination IA1+II+III showed a lower IIS activation than the ones incubated with IA1, II or III separately.

To further investigate IIS activation after incubation either with one phylotype individually or with mixture IA1+II+III, we performed ELISA assays. This way, we compared cytokines levels in supernatants for each condition. ELISA assays confirmed the IHC trends results in supernatants for IL-17, IL-10 and TGF-β (Fig. 4). Indeed, IL-17 concentration in supernatants was significantly decreased by the mixture IA1+II+III compared to IA1 only conditions ($p=0.0166$). The other tested pro-inflammatory cytokines IL-6 ($p=0.02652$) and IL-8, ($p=0.03423$) were also upregulated in supernatants from explants incubated with IA1 phylotype alone, in comparison with the combination IA1+II+III phylotypes (Fig. 4). Besides, the immunomodulator IL-10 showed a significant increase between both IA1 and IA1+II+III conditions ($p<0.001$) (Fig. 4). At the opposite, TGF-β was the only cytokine which did not demonstrate any significant modulation between all tested conditions, which is consistent with IHC results.

Taken together, IHC and ELISA results showed that restoring the phylotype diversity *in vitro* in a skin explant model is able to decrease the IIS activation in the explant comparing with incubation with phylotypes individually (IA1, II or III).

Discussion

Extensive crosstalks between the skin and its bacterial communities (microbiota) regulate local innate immune responses to ensure efficient skin functions and prevent cutaneous inflammation (2,7,32). Skin microbial communities are shaped by physiological characteristics and the individual leading to a complex equilibrium called skin microbial homeostasis. Recently, it has been shown that severe inflammatory acne (nodular) was associated with *C. acnes* phylotype diversity loss, with a high predominance of phylotype IA1 (26). Here, we report the loss of *C. acnes* phylotype diversity as a crucial mechanism capable of triggering skin inflammation. Thus, the association of different *C. acnes* phylotypes could prevent the development of inflammatory acne.

IIS is abnormally activated in acne patients

First of all, our study shows that the level of activation of IIS in normal skin of healthy subjects or with severe acne is not the same. This way, we confirm an upregulation of several IIMs in NLS from acne group comparing with healthy group. Interestingly, it was previously described that TLR-2 and IL-10 were weakly expressed in non-lesional skin from healthy subjects comparing with acne patients (34), which is consistent with our results. In the present study, TLR-2 and β-defensin-2 which are crucial IIS players, were significantly more expressed in NLS from acne group than NLS from healthy group. Previous works also described inflammation events in very early stage of acne lesion initiation (35). In another study, this abnormal IIS activation was previously suggested (3), as microbiological data only were not sufficient to explain the appearance of inflammatory diseases such as acne.

Moreover, local skin predispositions, even in non lesional skin condition were observed using a confocal microscopy approach exploring the sebaceous follicles in adult women with acne (36). These results show that there is a specific immunological pattern enabling the development of abnormal inflammatory reaction against a non-pathogenic bacterium such as *C. acnes*, favoring acne occurrence.

C. acnes subgroups diversity loss on the skin is a trigger for IIS activation

As the increase of IIS basal level activation observed in apparently normal skin of acne patients is not sufficient to explain the different levels of inflammatory severity observed in acne patients, we wanted to explore a worsening factor able to trigger the innate immunity of these patients. Previous studies reported the reduction of the microbiota diversity in inflammatory skin disease context, with most of the times, one genus taking advantage on the others (37,38). The most striking example is found in atopic dermatitis, with *Staphylococcus aureus* skin colonization inducing a dysbiosis (37,39). At the subgroup level, one study recently showed the drastic reduction of phylotype diversity in severe inflammatory acne (26). Thus, taking into account these data, we postulated that the drastic reduction of *C. acnes* phylotypes diversity previously showed, could trigger skin inflammation in a similar manner than in other cutaneous inflammatory disease as atopic dermatitis. Our data show that taken singly, *C. acnes* phylotype IA1, II or III induce a significant IIMs activation. Similar observations have been previously reported in different experimental models (16,25,31). In the present study, we demonstrate that the phylotype diversity loss on the skin is capable of triggering IIS activation, and thus the development of cutaneous inflammation. It suggests that *C. acnes* subpopulation diversity is a crucial factor for down regulating IIS activation *in vitro*. The present study also showed an increase in immune-modulator IL-10 after incubation with IA1 phylotype alone comparing with the combination IA1+II+III. Interestingly, it has

been previously mentioned that IL-10 high expression could favor the development of chronic inflammation maintained by the presence of *C. acnes*, and specifically resistant *C. acnes* strains (34). The chronic inflammation kept by IL-10 could play a central role in the development of nodular lesions. On the other hand, TLR-2 overexpression in keratinocytes after a contact with *C. acnes* has been previously reported (19). Recently, it has also been demonstrated that TLR-2 was able to recognize *C. acnes* CAMP Factor 1 from highly inflammatory strains (40), which is a crucial feature for host cell to induce skin inflammation in acne context. Moreover, in cell cultures, TLR-2 stimulation by *C. acnes* induces secretion of various cytokines (IL-8, IL-12), metalloproteinase MMP-9 and β-defensin-2 (19,41,42). In our study, the only marker that did not show any significant modulation with consistent results from IHC to ELISA assays, was TGF-β.

Interestingly, these data opens the debate to further questions. Indeed, it underlines the crucial role of the interactions between keratinocytes and microbiota, with a central role of the skin commensal *C. acnes* in acne. These interactions are sensed and controlled by the IIS mainly *via* PRRs (Pattern Recognition Receptors). As we observed an obvious upregulation of TLRs in acne skin (non-lesional) comparing with healthy controls, the abnormal inflammatory reaction facing *C. acnes* could be related to PRRs. TLRs represent a major part of PRRs, capable of recognizing PAMPs (Pathogen Associated Molecular Patterns) either through direct interaction or *via* an intermediate PAMP-binding molecule (43). Indeed, as a Gram-positive bacterium, *C. acnes* can be recognized by IIS through various PAMPs, including lipoteichoic acid, peptigoglycans, and triacyl lipopeptides (43).

Another communication mode was recently described: extracellular vesicles (EVs) are nowadays accepted to enable communication between host cells and bacteria, and *C. acnes* was recently described as a producer of EVs (44–46). Moreover, Choi *et al.* recently demonstrated that *C. acnes*-derived EVs were capable of promoting acne-Like phenotypes in

human epidermis, suggesting a possible role of *C. acnes* EVs in acne physiopathology (47). These results provide new paradigms for the mechanisms that trigger skin inflammation and they depict key data to develop future targets for innovative therapeutic approaches.

Our study shows that the inflammatory response observed in acne context, in addition to be dependent of *C. acnes* phylotype, is triggered by a loss of phylotype diversity at the surface of the skin. These results support the innovative idea that TLR-2 overexpression in patients with severe acne could explain the inappropriate inflammatory response observed against the skin commensal bacterium *C. acnes*, as it has been previously shown that TLR-2 was highly involved in *C. acnes* recognition and inflammation initiation (40,48). These data also support the idea that skin microbiota may be a crucial target in acne and thus, the interest of a therapeutically approach by topical modulators of microbiota both for maintaining and restoring the cutaneous diversity of *C. acnes* phylotypes. It also confirms that topical macrolides should be abandoned as they may participate to the loss of microbiota diversity, in addition to increase the bacterial resistance not only for *C. acnes* but also *S. epidermidis*, *S. aureus*, and *Streptococcus* (24,49–53).

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Author contributions

MA Dagnelie performed, designed the experiments and wrote the paper. S. Corvec contributed to interpretations and analysis of the results. M. Saint-Jean contributed to reageants/materials/analysis tools. JM Nguyen performed the statistical analysis. A. Khammari and B. Dréno were co-directors of this work, designed the study and contributed to results analysis. All authors reviewed and commented critically drafts of the manuscript for important intellectual content and gave final approval to submit for publication.

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Table legends

Table 1: Summary of *C. acnes* strains characterization and origins, used in the present study.

Table 2: Summary of antibodies used for immunohistochemistry experiments.

Figure legends

Figure 1: Innate immune markers studied in non-lesional skins (NLS) from acne (n=12) and healthy (n=13) groups. (a) Raw data of labelling intensity are presented according to the considered markers: IL-17, TGF-β, IL-1β, IL-10, TLR-2 and β-defensin-2. Data from acne and healthy groups were respectively combined and the variations are shown as mean +/- standard deviation *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Intensity labelling was evaluated using the following scale: null labelling (0), very weak labelling (1), weak labelling (2), moderate labelling (3), strong labelling (4). (b) The results are presented as the fold increase expression of IIMs comparing with NLS from healthy group.

Figure 2: Immunohistochemistry performed on healthy skin explants which have been in contact with phylotypes IA1, II and III singly or a mixture of IA1+II+III (n=3). The results from three donors were combined and the variation is shown as mean +/- standard deviation *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Slides were double-blinded read by two independent readers. Intensity labelling was evaluated using the following scale: null labelling (0), very weak labelling (1), weak labelling (2), moderate labelling (3), strong labelling (4). (a) Intensity labelling obtained after IHC experiments on three independent experiments, comparing explants after incubation with phylotype IA1, positive and negative controls. (b) Intensity labelling obtained after IHC experiments on three independent experiments, comparing explants after incubation with phylotype II, positive and negative controls. (c) Intensity labelling obtained after IHC experiments on three independent experiments, comparing explants after incubation with phylotype III, positive and negative controls. (d) Intensity labelling obtained after IHC experiments on three independent experiments, comparing explants after incubation with phylotype IA1, II, III and IA1+II+III to test whether the restoration of the phylotype diversity have an impact on the explant inflammatory

response. (e) Results of IHC labelling intensity scores, presented as fold increase/decrease taking IA1+II+III condition as reference.

Figure 3: Immunohistochemistry performed on skin explants after 6h-incubation with either *C. acnes* IA1, II or III phylotype alone, or with a combination of IA1+II+III phylotypes. The studied markers are innate immunity-related markers (TLR-2, β-defensin-2, IL-10, IL-17, IL-1β, and TGF-β). Negative control was obtained by incubating skin explant in culture media alone. Labelling appeared mainly in the epidermis for both markers. Intensity labelling was evaluated using the following scale: null labelling (0), very weak labelling (1), weak labelling (2), moderate labelling (3), strong labelling (4). Scale bars correspond to 300 μm.

Figure 4: Evaluation of cytokines concentrations released by normal skin explants after 6 h incubation with *C. acnes* phylotypes IA1, II or III singly, comparing with the combination of phylotypes IA1+II+III (n=3). Positive control was obtained by incubating skin explants with *E. coli* LPS, and negative control was obtained by incubating skin explant in culture media alone. The results from three donors were combined and the variation is shown as mean +/- standard deviation *** $p<0.001$. (a) IL-8 concentration (pg/mL), (b) IL-10 concentration (pg/mL), (c) IL-6 concentration (pg/mL), (d) IL-17 concentration (pg/mL), (e) TGF-β concentration (ng/mL).

Strain n#	Phylotype	Clonal Complex	SLST-type	Origin
1	IA1	CC18	A1	severe acne
2	II	CC53	K2	severe acne
3	III	CC43	L7	severe acne

Table 1: Summary of *C. acnes* strains characterization and origins, used in the present study.

Target	Manufacturer	Reference
TLR-2	SantaCruz	SC-10739
TGF-β	Serotec	MCA797
IL-17	Abcam	AB79056
IL-1β	Abcam	AB2105
IL-10	R&D Systems	AF-217-NA
β-Defensin-2	Abcam	AB63982

Table 2: Summary of antibodies used for immunohistochemistry experiments.