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# Pro-inflammatory activity of *Cutibacterium acnes* phylotype IA<sub>1</sub> and extracellular vesicles: An in vitro study

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## Abstract

Acne is a chronic inflammatory skin condition that involves *Cutibacterium acnes* (*C. acnes*), which is classified into six main phylotypes (IA<sub>1</sub>, IA<sub>2</sub>, IB, IC, II and III). Acne development is associated with loss of *C. acnes* phylotype diversity, characterised by overgrowth of phylotype IA<sub>1</sub> relative to other phylotypes. It was also shown that purified extracellular vesicles (EVs) secreted by *C. acnes* can induce an acne-like inflammatory response in skin models. We aimed to determine if the inflammatory profile of EVs secreted by *C. acnes* phylotype IA<sub>1</sub> from an inflammatory acne lesion was different from *C. acnes* phylotype IA<sub>1</sub> from normal skin, thus playing a direct role in the severity of inflammation. EVs were produced in vitro after culture of two clinical strains of *C. acnes* phylotype IA<sub>1</sub>, T5 from normal human skin and A47 from an inflammatory acne lesion, and then incubated with either human immortalised keratinocytes, HaCaT cells, or skin explants obtained from abdominoplasty. Subsequently, quantitative PCR (qPCR) was performed for human  $\beta$ -defensin 2 (hBD2), cathelicidin (LL-37), interleukin (IL)-1 $\beta$ , IL-6, IL-8, IL-17 $\alpha$  and IL-36 $\gamma$ , and ELISA for IL-6, IL-8 and IL-17 $\alpha$ . We found that EVs produced in vitro by *C. acnes* derived from inflammatory acne lesions significantly increased the pro-inflammatory cytokines and anti-microbial peptides at both transcriptional and protein levels compared with EVs derived from normal human skin. We show for the first time that *C. acnes* EVs from inflammatory acne play a crucial role in acne-associated inflammation in vitro and that *C. acnes* phylotype IA<sub>1</sub> collected from inflammatory acne lesion and normal skin produce different EVs and inflammatory profiles in vitro.

## KEYWORDS

acne, *Cutibacterium acnes*, extracellular vesicles, inflammation, skin

## 1 | INTRODUCTION

Acne vulgaris is a main dermatological concern, especially amongst adolescents and young adults, whose severity can negatively affect

quality of life and mental health. However, the underlying causes and pathogenesis of acne are still not entirely clarified, which would prevent effective treatment. Acne is considered a multifactorial disease that involves four major processes occurring in the

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pilosebaceous unit: dysseborrhoea, hyperkeratinization of the follicular duct, dysbiosis and innate immunity activation.<sup>1,2</sup>

It has been well documented that the commensal bacteria *C. acnes*, a Gram-positive anaerobic micro-organism formerly known as *Propionibacterium acnes*, is involved in acne development. *C. acnes* is classified into six main phylotypes (IA<sub>1</sub>, IA<sub>2</sub>, IB, IC, II and III) based on their biochemical, functional and genetic differences as assessed by DNA-based phylotyping approaches, such as multiplex touch PCR first demonstrated by Barnard et al.<sup>3,4</sup> Several other molecular tools have also been established to subclassify this bacterium, including single and multilocus sequence typing (SLST and MLST, respectively) and ribotyping, into clonal complex, sequence types and ribotypes.<sup>5-7</sup> Phylotype IA<sub>1</sub> is strongly associated with acne as an increase in this phylotype relative to the others was observed in acne biopsies, and co-culture of skin explants with phylotype IA<sub>1</sub> *C. acnes* can induce an inflammatory acne-like response in in vitro studies.<sup>5,8,9</sup> However, the switch between the commensal profile to the opportunistic pathogen of *C. acnes* IA<sub>1</sub> is still being explored and not yet fully understood. Recent studies have shown that acne development is associated with loss of *C. acnes* phylotype diversity leading to an enrichment in *C. acnes* phylotype IA<sub>1</sub>; in healthy skin of the face and back, *C. acnes* phylotype IA<sub>1</sub> accounts for approximately 41.6% and 36.3% as opposed to 84.4% and 95.6% in facial and back acne lesions, respectively.<sup>8,10</sup> Further, it has been shown that it is not the overall bacterial load, but a reduction in biodiversity, that is responsible for acne development; in vitro co-culture of skin models with the same number of mixed phylotype *C. acnes* bacteria is much less immunogenic than incubation with a single phylotype of *C. acnes*.<sup>8,10,11</sup> It has also been hypothesised that biofilm formation by this bacteria further aggravates acne by increasing bacterial adhesion to follicular keratinocytes, virulence factors and pro-inflammatory elements.<sup>12</sup>

Changes in microbial biodiversity at *C. acnes* phylotype level in favour of phylotype IA<sub>1</sub> lead to activation of the innate immunity whereby the bacterial antigenic products induce expression of pro-inflammatory molecules in keratinocytes as well as endogenous and circulating immune cells. Upregulation of pro-inflammatory cytokines such as interleukin (IL)-17 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and tumour necrosis factor (TNF)- $\alpha$ , as well as anti-microbial peptides such as human  $\beta$ -defensin 2 (hBD2) and cathelicidin anti-microbial peptide (LL-37), have been demonstrated in acne biopsies as well as in vitro skin models co-cultured with *C. acnes* bacteria.<sup>8,11</sup>

It has been suggested that not only the bacteria itself modulates acne, but also extracellular vesicles (EVs) produced by *C. acnes*. Recently, it was shown that *C. acnes*-derived EVs can induce a pro-inflammatory acne-like response in in vitro skin models.<sup>13-15</sup> EVs are secreted by bacteria as a form of communication as well as modulation of their surrounding. EVs from *C. acnes* contain lipids, glycolipoproteins, proteins, as well as other biologically active molecules, and they enter cells via receptor-mediated pathways, such as clathrin-dependent endocytosis.<sup>13</sup> Studies have also found that EVs from various *C. acnes* phylotypes are different in terms of lipid composition, size and protein composition as well as immunogenic capacity.<sup>16,17</sup>

This study was conducted to further investigate the differences in activation of the innate immunity by EVs derived from inflammatory acne lesions compared with EVs derived from normal skin *C. acnes*.

## 2 | METHODS

### 2.1 | Clinical isolates of *C. acnes* strains and culture conditions

Both clinical strains of *C. acnes*, T5 and A47 (nomenclature used at the CHU Nantes Bacteriology Department), were isolated from the skin of two different individuals. T5 was isolated from a healthy volunteer, and A47 from an inflammatory acne lesion (both phylotype IA<sub>1</sub>, clonal complex 18, SLST-type A1; Table S1). Bacteria were swabbed from human skin and streaked onto Schaedler agar plates containing 5% sheep's blood (bioMérieux, Marcy-l'Etoile, France). After confirmation of bacteria species identification and phylotype determination (by single locus sequence typing<sup>5</sup>), single colonies were inoculated into brain heart infusion (BHI) liquid medium containing 1% glucose and grown under static anaerobic conditions using the BD GasPak EZ system (Becton Dickinson, Franklin Lakes, NJ) at 37°C.

### 2.2 | Bacterial growth and isolation of EVs

Bacterial culture from a single colony was re-inoculated into fresh BHI + glucose medium at an OD of 0.1, and 10 days post-inoculation, the optical density was measured and the supernatant was harvested by centrifugation at 2000g for 10 min then 10000g for 30 min. The supernatant containing the EVs was first filtered with a 0.22  $\mu$ m filter, concentrated using Centricon Plus-70-10kDa (Merck, Darmstadt, Germany), and ultracentrifuged at 120000g for 3 h at 4°C. The pelleted EVs were washed with phosphate-buffered saline (PBS) via ultracentrifugation at 120000g for 1 h at 4°C, then resuspended in PBS, quantified by the BCA protein analysis kit (Pierce, Thermo Fisher Scientific, Waltham, MA), and stored at -80°C until analysis.

### 2.3 | Scanning and transmission electron microscopy and measurement of EV size

Purified EVs (5  $\mu$ L) in PBS were dropped on glow-discharged 200-mesh carbon coated copper grids and then let adsorbed for 20 min followed by fixation with 0.08% glutaraldehyde for 5 min. After several washes with distilled water, the EV-attached grids underwent contrast staining with UranylLess (Mauressac, France), air-dried overnight, and coated with palladium (less than 1 nm thickness). The grids were observed with scanning and transmission electron microscopy (STEM) detector on a SEM Zeiss GeminiSEM 300. Purified EVs from each *C. acnes* strain (T5 and A47) from four different experiments were visualized by STEM. At least five images of each

sample were analysed, and EV size was measured using the Fiji/ImageJ software.<sup>18</sup>

## 2.4 | HaCaT keratinocyte cell line and skin explants

Immortalised keratinocytes, HaCaT cells, were purchased from Cell Line Services (Hamburg, Germany) and cultured in DMEM (Thermo Fisher) with 10% fetal bovine serum (Eurobio, Essonnes, France) and 1% Glutamax (Invitrogen, Carlsbad, CA) at 37°C with 5% CO<sub>2</sub>, as per the distributor's recommendations.

Skin explants from abdominoplastic surgery were furnished by the Department of Plastic Surgery, Reconstructive, and Aesthetic Surgery at the University Hospital of Nantes. On the day of the surgery, the skin explant was placed immediately on ice, the fat was removed, and 6 mm biopsy punches (Kai Europe, Solingen, Germany) were made and immediately placed in DMEM under different treatment conditions.

## 2.5 | Treatment of HaCaT cell line and explants with *C. acnes* EVs

HaCaT cells and biopsy punches were treated with 10 µg/mL of A47 or T5 EVs, while the sham-treated control cells received the same volume of PBS. At 24 h post-treatment, the supernatant was stored at -80°C until analysis, and the cells or tissues were lysed for RNA extraction.

## 2.6 | RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted from HaCaT cells and skin explants using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions. cDNA was synthesized using Superscript III (Invitrogen) as per the manufacturer's protocol.

qPCR targeting hBD2, LL-37, IL-1β, IL-6, IL-8, IL-17α and IL-36γ was performed with KicqStart SYBR Green primers (Table S2) from Sigma-Aldrich (St. Louis, MI) using the PowerTrack SYBR Green mastermix (Thermo Fisher) according to the manufacturer's instructions. qPCR was run in a CFX96 Bio-Rad Thermocycler (Hercules, CA) under the following conditions: 95°C for 5 min, 40 cycles of 95°C for 10 s and 58°C for 10 s, followed by a melt curve. Analysis was performed with the online program, 'Do my qPCR calculation'.<sup>19</sup>

## 2.7 | ELISA in HaCaT cell line and explants

The supernatants from treated HaCaT cells and explants were subjected to ELISA for IL-17α, IL-6 and IL-8 (Invitrogen) according to the

manufacturer's protocol, and data were normalised to the vehicle control.

## 2.8 | Statistical analysis

All statistical analyses were performed using GraphPad Prism V.9 software (Dotmatics, Boston, MA). Comparisons were performed with Student's t-test or one-way ANOVA with post-hoc Tukey test as necessary. Statistical significance was considered when  $p < 0.05$ .

# 3 | RESULTS

## 3.1 | Culture of *C. acnes* strains to obtain EVs

Figure 1A showed that the T5 strain (healthy volunteer) grew significantly faster in the BHI culture medium supplemented with glucose than the A47 strain (acne lesion). At 10 days post-inoculation, the OD of the T5 strain was  $1.88 \pm 0.12$  as compared to  $0.71 \pm 0.08$  of A47 ( $p = 0.0001$ ).

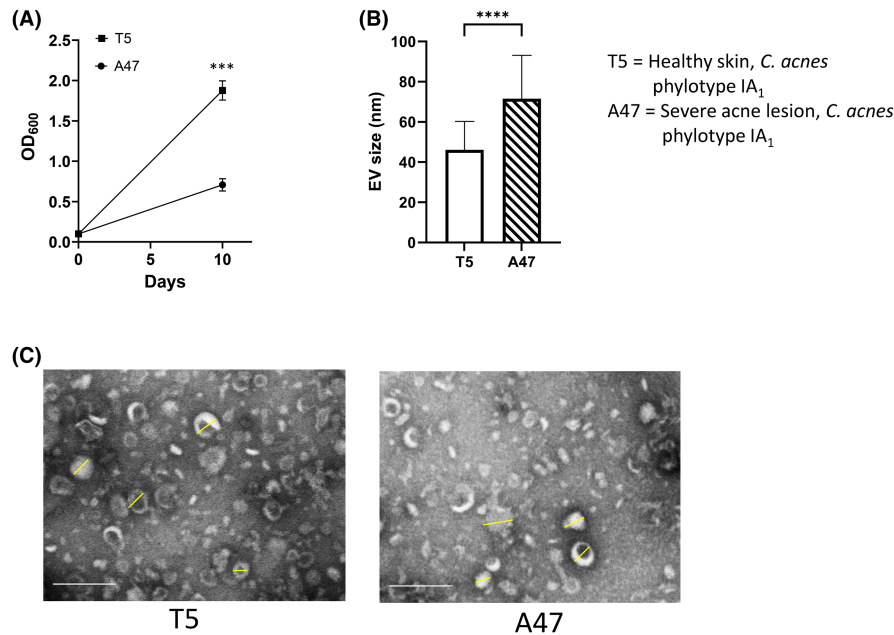
## 3.2 | Characterization of EVs

The isolated EVs from the two strains were visualised by STEM. The average size of the EVs was determined, and Figure 1B,C shows that EVs from A47 ( $71.5 \pm 21.6$  nm, range 31.9–175.2 nm) were significantly larger than those of T5 ( $46.1 \pm 14.1$  nm, range 20.4–102.0 nm,  $p < 0.0001$ ).

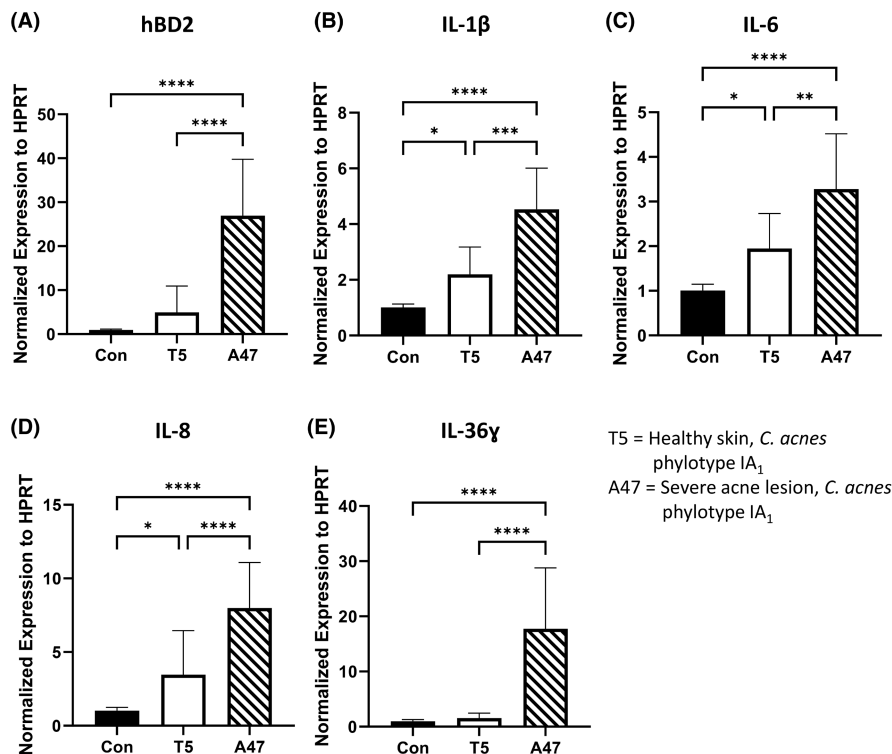
## 3.3 | Transcriptional level of pro-inflammatory markers in HaCaT cell line and explants

Using an immortalised cell line of keratinocytes (HaCaT; Figure 2A–E) as an in vitro model, it was shown that A47 EVs, as compared to vehicle control (PBS) and T5 EVs, induced a significant and dramatic upregulation of the different markers at the transcriptional level as summarized in Table S3. On the other hand, T5 EVs induced either no or only a slight upregulation in the pro-inflammatory markers as compared to vehicle controls. IL-17α gene expression was not detected in HaCaT cells.

We compared the transcript level of the pro-inflammatory markers induced by EVs from acne-associated *C. acnes* (A47) to that from normal skin *C. acnes* (T5) to determine if there was a difference. We found that all immune markers were significantly upregulated in the A47 EV-treated HaCaT cell line as compared to T5 EV-treated cells: hBD2 ( $p < 0.0001$ ), IL-1β ( $p = 0.0001$ ), IL-6 ( $p = 0.002$ ), IL-8 ( $p < 0.0001$ ), and IL-36γ ( $p < 0.0001$ ). LL-37 was not expressed by HaCaT cells.



**FIGURE 1** Growth and morphological differences between the two different clinical isolates of *Cutibacterium acnes* (*C. acnes*): T5 (phylogroup IA<sub>1</sub>, normal skin) and A47 (phylogroup IA<sub>1</sub>, lesional acne). (A) Growth analysis of *C. acnes* strains T5 (phylogroup IA<sub>1</sub>, normal skin) and A47 (phylogroup IA<sub>1</sub>, acne lesion). Bacterial cultures of T5 and A47 were inoculated into fresh medium at an optical density (OD) of 0.1, and then OD measurements of the bacterial cultures were taken at 10 days post-inoculation. (B) EVs derived from *C. acnes* T5 and A47 were subjected to scanning transmission electron microscopy (STEM), and EV size was measured from at least five images from each sample. (C) Images of EVs derived from *C. acnes* T5 and A47 by SEM with STEM detector. Experiments were performed at least three times in triplicate. Significance was considered when *p* < 0.05 as determined by Student's *t*-test. \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

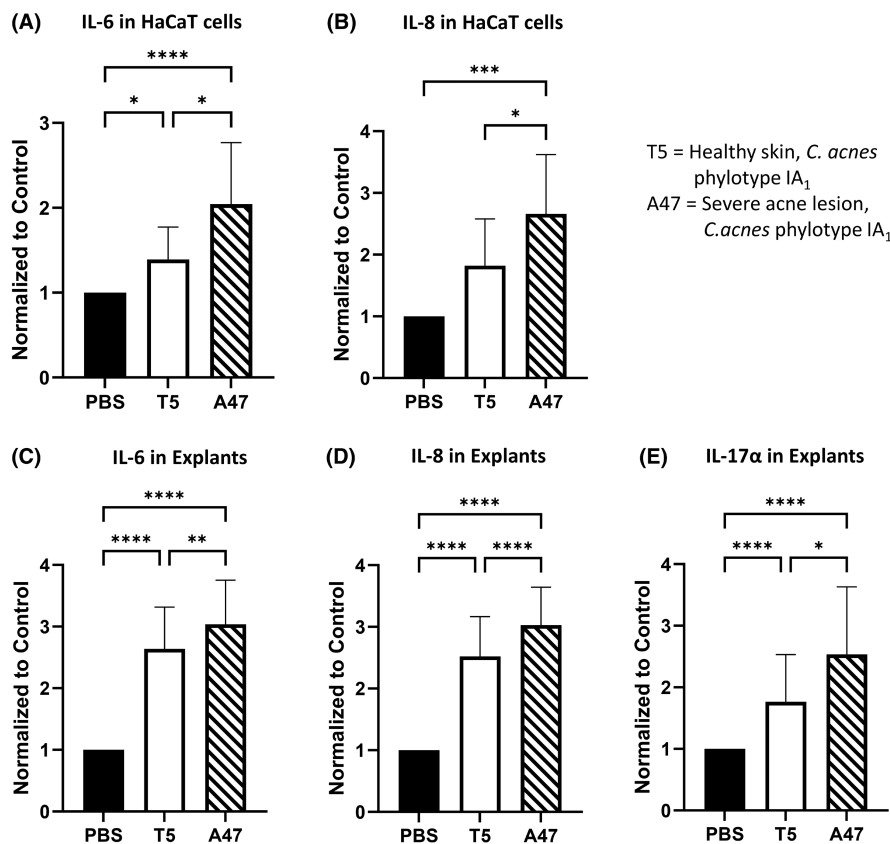


**FIGURE 2** Transcriptional analysis by qPCR for hBD2, IL-1β, IL-6, IL-8 and IL-36γ in extracellular vesicles (EVs)-treated immortalised human keratinocytes (HaCaT cells). qPCR for innate immune markers (A) hBD2, (B) IL-1β, (C) IL-6, (D) IL-8 and (E) IL-36γ in HaCaT cells treated for 24 h with EVs isolated from *Cutibacterium acnes* strains T5 (phylogroup IA<sub>1</sub>, normal skin) and A47 (phylogroup IA<sub>1</sub>, acne skin). Vehicle control was the incubation of cells with the same volume of phosphate-buffered saline (PBS). Experiments were performed at least three times in triplicate. Significance was considered when *p* < 0.05 as determined by multiple comparisons using ANOVA analysis. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

In an ex vivo human skin explant model (Figure 3A–F; Table S3), we also found that as compared to T5 EVs, A47 EVs induced significant increases in hBD2 (*p* = 0.005), LL-37 (*p* = 0.006), IL-1β (*p* = 0.02), IL-6 (*p* = 0.003), IL-8 (*p* = 0.02), IL-17α (*p* = 0.002) and IL-36γ (*p* = 0.04)

(Figure 3A–F). T5 EVs also upregulated IL-8 (*p* = 0.04) as compared to vehicle control (PBS) although to a lesser extent than the increase induced by A47 EVs. These results corroborated those obtained in the HaCaT cell line.





**FIGURE 4** Protein analysis by ELISA for innate immune markers IL-6, IL-8 and IL-17 $\alpha$  in extracellular vesicles (EVs)-treated immortalized human keratinocytes (HaCaT cells) and human skin explants. ELISA for innate immune markers (A) IL-6 and (B) IL-8 in HaCaT cells, and (C) IL-6, (D) IL-8, and (E) IL-17 $\alpha$  in human skin explants treated for 24 h with EVs derived from *Cutibacterium acnes* (*C. acnes*) strains T5 (phylotype IA<sub>1</sub>, normal skin) and A47 (phylotype IA<sub>1</sub>, acne skin). Experiments were performed at least three times in triplicate. Significance was considered when  $p < 0.05$  as determined by multiple comparisons using ANOVA analysis. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

mediated by secreted EVs, which was thought to be not possible in Gram-positive bacteria due to their thick, impenetrable cell wall.<sup>20</sup> However, in recent years, there has been increasing knowledge on the biogenesis and secretion of EVs in Gram-positive bacteria.<sup>21–23</sup> Several recent studies have shown that *C. acnes* bacteria can produce and secrete EVs, and a comparison of the EVs from various phylotypes showed that they harbour different size and content profiles.<sup>14,16,17</sup> In this study, we found that the healthy skin-associated *C. acnes* IA<sub>1</sub> strain (T5) produced significantly smaller EVs than those found in the acne-derived *C. acnes* IA<sub>1</sub> strain (A47) (Figure 1B,C), which suggests that EV size is not only phylotype-specific, but also dependent on the skin condition and bacterial strain. *C. acnes* EVs have been demonstrated to enter human cells through a clathrin-mediated endocytosis pathway, thus allowing for the transfer of bacterial cargo, including potentially proteins, lipids, DNA, RNA as well as other biomolecules, into the host.<sup>13</sup> In this context, the difference in size between T5- and A47-derived EVs is likely due to a difference in the cargo. Chudzick et al. found that the amount of lipids present in the EVs was different between the different strains; six types of lipids were obtained from phylotype IA<sub>1</sub>, eight from phylotype II, and 11 from phylotype IB.<sup>17</sup> They further showed that phylotype IA<sub>1</sub> EVs contained proteins in the range of 15–75 kDa, while the EVs of phylotypes II and IB were 37–75 kDa and around 20 kDa, respectively. Cros et al. also found differences at the proteomic level between the EVs from the phylotype IA<sub>1</sub> and two phylotype IB strains; phylotype IA<sub>1</sub> EVs express a significantly larger repertoire of unique proteins (32.2%) as compared to those from the phylotype

IB (8.1 and 3.4%), in addition, the phylotype IA<sub>1</sub> vesicular proteins predominantly participates in biosynthetic and metabolic processes while those of phylotype IB strains are mostly related to transport.<sup>16</sup> Future studies to examine the differences in EV cargo load between our two strains would further enlighten the mechanisms by which EVs contribute to the differences between the two phylotype IA<sub>1</sub> strains studied here (normal skin and acne lesion).

Emerging evidence has demonstrated that activation of innate immunity by *C. acnes* is pertinent in acne development as the early steps to inflammation.<sup>24</sup> Many studies have compared the different phylotypes of *C. acnes* and showed that acne-associated strains can upregulate innate immunity; treatment of human cells with live *C. acnes* bacteria or extracts can induce pro-inflammatory cytokines, such as IL-17 $\alpha$ , IL-8, TNF $\alpha$ , IL-1 $\beta$ , IL-6 and TGF- $\beta$ , as well as anti-microbial peptides, including hBD2 and LL-37, and immune-regulating molecules, such as matrix metalloproteinases (MMPs).<sup>8,25–27</sup> However, recent evidence shows that not only the bacteria can elicit an immune response, but their secreted EVs can also induce the expression of pro-inflammatory molecules.

Recent studies have used purified EVs from *C. acnes* obtained from acne lesions to treat several human skin models which demonstrated that they also have immune-inducing properties. It was shown that EVs secreted by *C. acnes* can induce numerous innate immune markers, including IL-17 $\alpha$ , IL-8, GM-CSF, CXCL-1, CXCL-5, TNF $\alpha$ , IL-1 $\beta$  and IL-6, suggesting that EVs are a potential pathway for *C. acnes* to regulate inflammation during acne development.<sup>13,16,28</sup> Although Cros et al. demonstrated that EVs from a phylotype IA<sub>1</sub>

clinical isolate strain induced a larger inflammatory response as compared to those derived from phylotype IB strains, no study thus far has compared differences in the EVs between strains of the same phylotype from inflammatory acne lesion and healthy skin. Our results obtained in this study confirmed that there was an upregulation in the pro-inflammatory markers, IL-17 $\alpha$ , IL-8, IL-1 $\beta$ , IL-6 and IL-36 $\gamma$ , as well as in the anti-microbial peptides, hBD2 and LL-37, in both skin models induced by EVs isolated from the acne-associated IA<sub>1</sub> strain (A47) compared with those obtained from *C. acnes* IA<sub>1</sub> found in normal skin (Figures 2–4). Since the phylotype IA<sub>1</sub> strain was found to have increased immune activation potential, biofilm formation capability, as well as expression of genes for lipid transport, metabolism and virulence factors compared with other phylotypes, this suggests that the relative overabundance of the phylotype IA<sub>1</sub> strain in acne lesions is associated with a modification of the virulent profile of *C. acnes* IA<sub>1</sub>.<sup>29–34</sup> For example, it was found that *C. acnes* clinical isolates associated with a high inflammatory potential express a putative virulent gene, CAMP factor 1, which could be recognized by toll-like receptor 2 to provoke an immune response.<sup>35,36</sup> Additionally, *C. acnes* isolates from human skin that could provoke a strong immune response were demonstrated to harbour a linear plasmid that appeared to express many cytokine modulatory elements by gene ontology analysis.<sup>37</sup> Modulation of the virulence of *C. acnes* could also be induced by the local microenvironment, mainly sebum which presents both qualitative and quantitative modifications in acne, taking into account the results of Borrel et al. which showed that an acneic strain of *C. acnes* had modified proliferation and IL-8 expression when cultured in a sebum-like medium.<sup>38</sup> Thus, our hypothesis is that modification of the sebum in the environment of *C. acnes* (pilosebaceous follicle) in acne lesions induces activation of virulence factors in *C. acnes* IA<sub>1</sub> and consequently, EVs derived from these *C. acnes* IA<sub>1</sub> could contain a higher amount of virulence factors. Our data demonstrate that there is a significant difference in the inflammatory profile of the EVs from the two different strains which may be due to changes in virulence. A comparison of the EV contents would throw light on the causes of the differences in the inflammatory profiles between the two strains.

A limitation of our study is that we compared a single strain each from a healthy volunteer and an inflammatory acne lesion, and a larger study comparing multiple strains of the same phylotype would further validate our results. Further, while the homogeneity and size of the EVs were determined using STEM, the purity and possible contamination with other *C. acnes* virulence factors were not elucidated.

In conclusion, we show here that there is a difference in the pro-inflammatory profiles induced in a keratinocyte cell line and ex vivo skin explants by EVs produced in vitro by *C. acnes* derived from acne lesions and normal skin. Our study is a proof of concept that differences in profile exist between strains of the same phylotype derived from healthy and acne skin. Our results identify an additional factor that could contribute to the difference in inflammatory potential between *C. acnes* derived from healthy skin and severely inflamed acne lesions.

## AUTHOR CONTRIBUTIONS

C.T.C. performed the experiments, analysed the data, and wrote the manuscript. U.L. provided the human skin explants and scientific input. S.C. provided the bacteria and scientific input. V.M. and C.M. provided scientific input and contributed to the analysis. J.V. performed the STEM. A.K. and B.D. designed the study, provided scientific input, contributed to the analysis, and oversaw the project. All authors critically reviewed the manuscript and approved the final submission.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted with funding from industrial sources.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author. All data are available upon request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1:** Properties of the *Cutibacterium acnes* clinical isolates used in this study.

**Table S2:** Kicqstart primers used in this study.

**Table S3:** Summary of gene expression of hBD2, LL-37, IL-1 $\beta$ , IL-6, IL-8, IL-17 $\alpha$ , and IL-36 $\gamma$  in EV-treated HaCaT cell line and skin explants.

**Table S4:** Summary of protein expression of IL-6, IL-8 and IL-17 $\alpha$  in EV-treated HaCaT cell line and skin explants.

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