The Anti-Inflammatory Activities of *Propionibacterium acnes* CAMP Factor-Targeted Acne Vaccines

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Inflammatory acne vulgaris afflicts hundreds of millions of people globally. *Propionibacterium acnes*, an opportunistic skin bacterium, has been linked to the pathogenesis of acne vulgaris. Our results show that a secretory Christie-Atkins-Munch-Petersen (CAMP) factor of *P. acnes* is up-regulated in anaerobic cultures. Mutation of CAMP factor significantly diminishes *P. acnes* colonization and inflammation in mice, demonstrating the essential role of CAMP factor in the cytotoxicity of *P. acnes*. Vaccination of mice with CAMP factor considerably reduced the growth of *P. acnes* and production of MIP-2, a murine counterpart of human IL-8. Acne lesions were collected from patients to establish an ex vivo acne model for validation of the efficacy of CAMP factor antibodies in the neutralization of the acne inflammatory response. The *P. acnes* CAMP factor and two proinflammatory cytokines (IL-8 and IL-1β) were expressed at higher levels in acne lesions than those in nonlesional skin. Incubation of ex vivo acne explants with monoclonal antibodies to CAMP factor markedly attenuated the amounts of IL-8 and IL-1β. Our work using an ex vivo acne model shows that *P. acnes* CAMP factor is an essential source of inflammation in acne vulgaris.

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INTRODUCTION

Propionibacterium acnes, now referred as *Cutibacterium acnes* based on a new taxonomic classification (Boisrenoult, 2018), is a Gram-positive skin commensal bacterium and predominates (>60% of total bacteria) the facial skin in humans (Grice et al., 2009). Nearly everyone hosts *P. acnes* (Ahn et al., 1996; Brook and Frazier, 1991), which makes up almost half of the total skin microbiome (Tancrede, 1992), with an estimated density of 10^2 to 10^{5-6} cm^2 (Leyden et al., 1998; McGinley et al., 1978). Overgrowth of *P. acnes* has been linked to acne vulgaris, a skin disease afflicting more than 85% of teenagers and over 40 million people in the United States (Fried and Wechsler, 2006; Taglietti et al., 2008; White, 1998). Current treatments for acne are often inadequate or difficult to tolerate, but many therapies target the *P. acnes* organism, which has been implicated in the genesis of inflammation in acne (Dessinioti and Katsambas, 2010; Leyden, 2001). Here, we used a vaccination approach to test whether *P. acnes* Christie-Atkins-Munch-Petersen (CAMP) factor, a secretory virulence factor (Valanne et al., 2005), is a main source of inflammation in acne vulgaris.

Five genes with approximately 32% sequence homology to the co-hemolytic CAMP factor of *Streptococcus agalactiae* were detected in the *P. acnes* genome (Bruggemann, 2005). *P. acnes* CAMP factor is able to bind to immunoglobulin G and M classes and acts as a pore-forming toxin (Valanne et al., 2005). Previous data from our laboratory showed that CAMP factor combined with sphingomyelinase exerts the co-hemolytic activity that can confer cytotoxicity to both keratinocytes and macrophages (Nakatsuji et al., 2011), enhancing virulence by degrading and invading host cells. Furthermore, *P. acnes* CAMP factor can induce cell death of sebocytes in sebaceous glands and trigger inflammation (Liu et al., 2011). In addition, it has been shown that *P. acnes* targets skin cells, namely, keratinocytes and phagocytic cells like macrophages, stimulating the cells to produce proinflammatory cytokines, including IL-8, IL-1β, IL-12, and tumor necrosis factor-α, leading to the inflammation in acne vulgaris (Contassot and French, 2014; Kurokawa et al., 2009; Nagy et al., 2006; Nakatsuji et al., 2011; Qin et al., 2014).

Antibiotics for acne treatment may run a risk of developing resistant bacteria and have no capability of neutralizing the secretory toxins. Isotretinoin, 13-*cis*-retinoic acid, has been extensively prescribed for the systemic treatment of severe acne (Layton et al., 2006). However, isotretinoin can cause depression and an increased rate of birth defects if taken during pregnancy (Mondal et al., 2017). Thus, the drug is highly regulated by the US Food and Drug Administration. Despite the success of isotretinoin in the treatment of acne vulgaris, there remains no effective modality that can prevent

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Abbreviations: CAMP, Christie-Atkins-Munch-Petersen; GFP, green fluorescent protein; mAb, monoclonal antibody; TGF, transforming growth factor; Th, T helper

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the occurrence of acne vulgaris. We have previously shown that the vaccination approach prevents the inflammation caused by *P. acnes* CAMP factor (Huang et al., 2008; Kao and Huang, 2009; Liu et al., 2011; Lo et al., 2011; Nakatsuji et al., 2008a, 2008b, 2008c, 2008d, 2011). Results from our previous publications illustrated that vaccination using surface sialidase (Nakatsuji et al., 2008b) or heat-killed *P. acnes* (Nakatsuji et al., 2008a) as an antigen significantly suppressed *P. acnes*-induced inflammation. Specifically, immunized mice had a reduction in ear swelling and production of MIP-2. Here, we explored the use of a specific secretory virulence factor as a vaccine because it has been reported that specific inhibition of secretory virulence factors presents less selective pressure for the development of resistant bacteria (Rasko et al., 2008). We have previously shown that CAMP factor of *P. acnes* is immunogenic and a secretory toxin (Nakatsuji et al., 2011) and that vaccination of mice with *Escherichia coli* overexpressing CAMP factor provides therapeutic protection against *P. acnes* (Liu et al., 2011; Lo et al., 2011; Nakatsuji et al., 2011). With the goal of vaccination approaches for acne treatments in the future, we decided to further assess the relationship of *P. acnes* in humans. These experiments included vaccination with a clinical aluminum adjuvant, consideration of the status of *P. acnes* as commensal bacteria in humans, and evaluation of the effectiveness of antibodies to CAMP factor using human ex vivo acne explants.

RESULTS

Up-regulation of *P. acnes* CAMP factor under anaerobic conditions

P. acnes is an anaerobic microbe endemic in humans. The genome of *P. acnes* has revealed genes for many virulence factors involved in degrading host tissues and genes involved in the induction of inflammation (Bruggemann, 2005; Schaefer et al., 1980). It has been shown that the production of virulence factors of *P. acnes* was markedly increased in the absence of oxygen (Cove et al., 1983). These virulence factors, which are either secreted from *P. acnes* or anchored in the cell wall, stimulate adjacent host cells and trigger inflammation and cell damage. In an effort to identify the virulence factors that are highly expressed under anaerobic conditions, a quantitative proteomic analysis was performed on *P. acnes* in the presence or absence of oxygen. Changes in the abundances of individual protein species in *P. acnes* grown with/without oxygen were analyzed using the non−gel-based isotope-coded protein label method (Schmidt et al., 2005). A total of 342 proteins were sequenced by nano-liquid chromatography linear ion trap quadrupole tandem mass spectrometry. Of these, 23 proteins were identified as up- or down-regulated under anaerobic or aerobic conditions (see Supplementary Table S1 online). By quantification of an internal peptide, DLLKAAFDLR, of CAMP factor (accession number: WP_002518322), we found that the anaerobic culture led to an approximately 1.5-fold increase in the expression of CAMP factor (Figure 1a). The internal peptide was sequenced and is presented in Figure 1b.

The essential role of CAMP factor in *P. acnes*-induced inflammation

Although results in our previous publication (Huang et al., 2008) showed that sera from CAMP factor-immunized mice were able to neutralize the virulence of CAMP factor, a knock-out mutant (Δ*camp*) of *P. acnes* CAMP factor (Sorensen et al., 2010) was used to verify the essential role of CAMP factor in *P. acnes*-induced inflammation. We first examined the in vitro growth of Δ*camp*. The Δ*camp* and wild-type *P. acnes* (266; 1-1a, ST18) were grown in Reinforced Clostridium Medium (BD, Sparks, Baltimore, MD) in a 96-well microplate under anaerobic conditions. The optical density$_{600}$ was read every day. We found that both Δ*camp* and wild-type *P. acnes* bacteria have the same growth rates, indicating that mutation of CAMP factor did not affect its growth in vitro (data not shown). To investigate the essential role of CAMP factor in *P. acnes*-induced inflammation, ears of Institute of Cancer Research (Harlan Labs, Placentia, CA) mice were injected intradermally with live Δ*camp* (10^7 colony-forming units in 20 μl phosphate buffered saline) or wild-type *P. acnes*. Injection of the same amount of phosphate buffered saline served as a control. Compared with injection with wild-type *P. acnes*, injection of mice with Δ*camp* caused less swelling (data not shown), redness (Figure 1c), and secretion of MIP-2 (Figure 1d). Furthermore, the mouse ear injected with Δ*camp* resulted in significantly less *P. acnes* growth than those injected with wild-type *P. acnes* (Figure 1e and f). These results show the requirement of CAMP factor for *P. acnes*-induced inflammation.

Detectable but low titers of antibodies to CAMP factor in acne patients

Previous findings have shown that acne patients produce a higher titer of antibody to *P. acnes* bacteria than individuals without acne (Basal et al., 2004). Here, we conducted ELISA to determine the titer of antibody to *P. acnes* CAMP factor. As shown in Figure 2a, the titer (1:400) of antibody to CAMP factor in healthy individuals was low but still detectable and was also detected in acne patients at a titer of 1:800. To determine whether antibodies to CAMP factor in humans at these low titers have the ability to reduce the cytotoxicity of *P. acnes*, murine RAW264.7 macrophage cells were incubated with *P. acnes* ATCC 6919 overnight in the presence of different titers of inactivated sera 2.5% (volume/volume) obtained from healthy individuals and acne patients with titers of antibody to CAMP factor at 1:400 and 1:800. Cells were also incubated with *P. acnes* in the presence of inactivated sera collected from mice vaccinated with UV-inactivated *E. coli* overexpressing CAMP factor with titers of antibodies to CAMP factor ranging from 1:500 to 1:7,821,500. Cells incubated with *P. acnes* and inactivated sera obtained from mice vaccinated with UV-inactivated *E. coli* overexpressing green fluorescent protein (GFP) served as a negative control. As shown in Figure 2b, human sera with a titer of antibody to CAMP factor at 1:800 were unable to abrogate the *P. acnes*-induced cell death. Cell death was significantly inhibited ($>$24%) only when the anti-CAMP factor mouse sera with titers greater than 1:62,500 were used, suggesting that the low titers of antibodies to CAMP factor produced in humans are not sufficient to reduce the cytotoxicity of *P. acnes*.

Protective immunity against *P. acnes* conferred by vaccination with recombinant CAMP factor

Institute of Cancer Research mice were subcutaneously vaccinated with recombinant CAMP factor or green

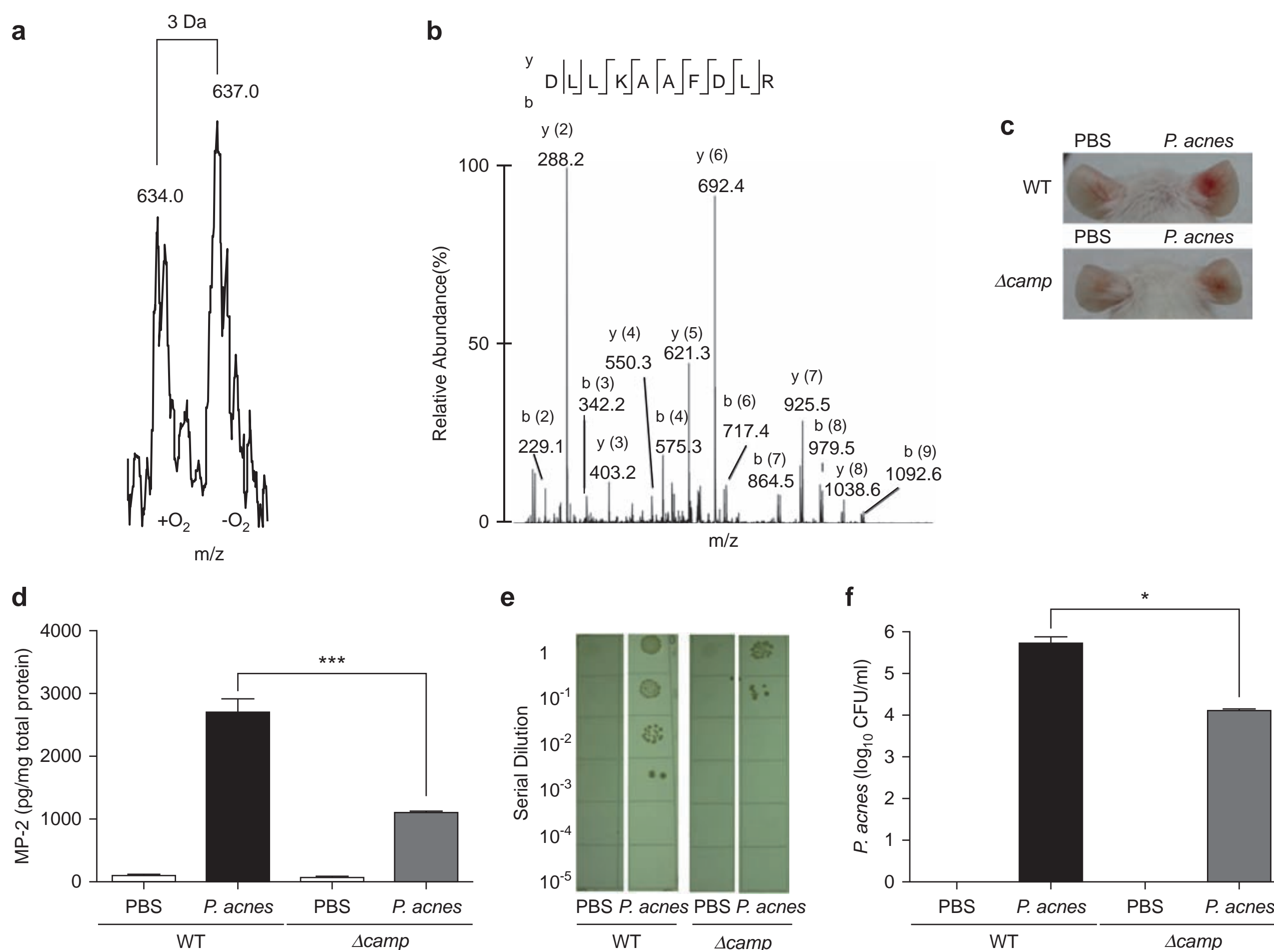

Figure 1. Up-regulation of *Propionibacterium acnes* CAMP factor under anaerobic conditions and the essential role of CAMP factor in *P. acnes*-induced proinflammatory MIP-2 cytokine and bacterial colonization. (**a**) *P. acnes* ATCC 6919 was grown under aerobic and anaerobic conditions. Lysates (1 mg) of *P. acnes* (ATCC 6919) from aerobic and anaerobic growth were labeled with $^{12}C_6$-Nic-NHS and $^{13}C_6$-Nic-NHS, respectively. After mixing $^{12}C_6$-Nic-NHS— and $^{13}C_6$-Nic-NHS—labeled samples, the mixture was subjected to an LC-LTQ mass spectrometer. CAMP factor with a mass difference of 3 Da per labeled site in mass spectra is shown. (**b**) A peptide, DLLKAAFDLR, was sequenced and assigned to the internal peptide of CAMP factor. (**c**) The right ears of Institute of Cancer Research (London, UK) mice were injected intradermally with a wild-type or CAMP factor-mutant *P. acnes* strain (*Δcamp2*) (10^7 CFU in 20 μl PBS). Injection of 20 μl PBS into left ears served as a control. (**d**) Three days after injection, the proinflammatory MIP-2 cytokines were quantified by ELISA. (**e, f**) The bacterial colonies (CFUs) were enumerated by plating serial dilutions ($1:10^1$—$1:10^5$) of the ear homogenate on an agar plate. Error bars represent mean ± standard deviation of five mice. $^*P < 0.05$, $^{***}P < 0.001$ by Student *t* test. CAMP, Christie-Atkins-Munch-Petersen; CFU, colony-forming unit; LC-LTQ, liquid chromatography linear ion trap quadrupole; Nic-NHS, *N*-nicotinoyloxy-succinimide; PBS, phosphate buffered saline; WT, wild type.

fluorescent protein (50 μg) as antigens without addition of an exogenous adjuvant and boosted with the same amount antigen 2 weeks after first vaccination. The production of antibody against CAMP factor was detected by ELISA assay 5 weeks after first vaccination. Antibodies to CAMP factor were produced in mice vaccinated with recombinant CAMP factor alone at a titer of approximately 1:800,000 (Figure 3a). The result indicates that *P. acnes* CAMP factor is immunogenic by itself. However, because of the possibility of development of a human vaccine, aluminum, a common adjuvant for human use (Lindblad, 2004), was selected as an adjuvant. With the addition of aluminium, there was more than a 4-fold increase in the titers of antibodies to CAMP factor, when mice were vaccinated with recombinant CAMP factor (50 μg) along with 2% aluminum.

To determine if CAMP factor vaccination can decrease *P. acnes*-induced inflammation, the right ear of each vaccinated mouse was intradermally injected with 20 μl of *P. acnes* (10^7 colony-forming units), and the left ear was injected with 20 μl of phosphate buffered saline as a control (Figure 3b) 5 weeks after first vaccination. Compared with mice immunized with green fluorescent protein, mice vaccinated with recombinant CAMP factor alone without aluminum decreased *P. acnes*-induced erythema and ear thickness (Figure 3b). In addition, vaccination with CAMP factor alone markedly reduced the production of proinflammatory MIP-2 cytokine (Figure 3c) and led to one log_{10} reduction in *P. acnes* colonization in ear skin (Figure 3d). When the titers of antibodies to CAMP factor were augmented by 2% aluminum, the reduction in ear thickness and MIP-2 production (5,109 ± 212.1 to 511 ± 5.9 pg/ml) was considerably enhanced (Figure 3b and c), and two log_{10} reduction in *P. acnes* colonization in ear skin was detected (Figure 3d). The titers of antibodies to CAMP factor in mice vaccinated with 10 or 20 μg recombinant CAMP factor plus 2% aluminum can reach the levels at 1:512,500 and

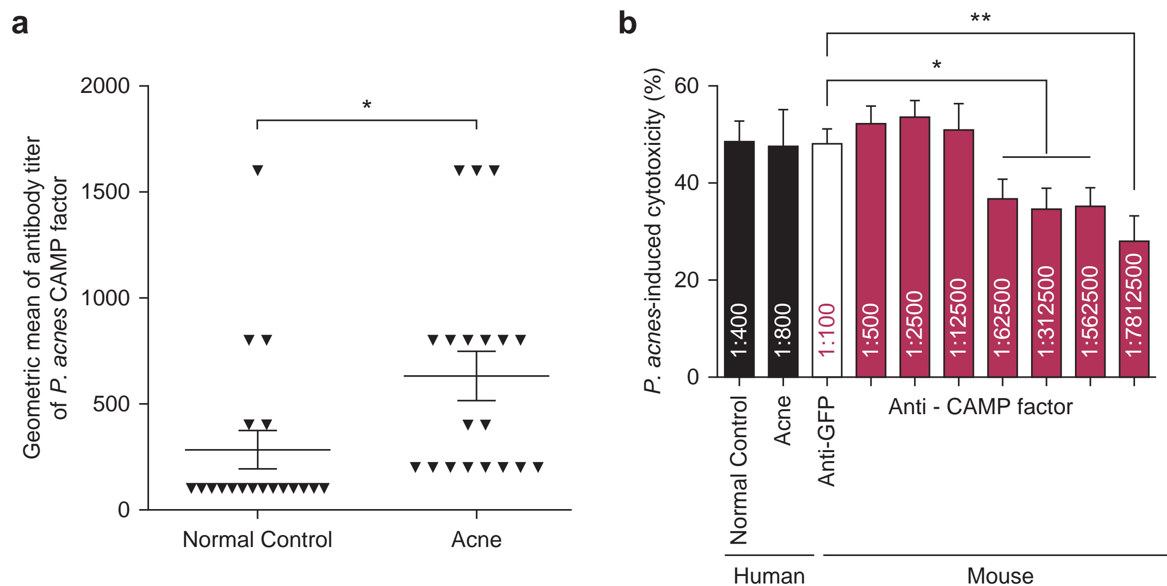

Figure 2. The detectable antibodies to CAMP factor in acne patients and reduction of cytotoxicity of *Propionibacterium acnes* to macrophages by high titers of antibodies generated in the *Escherichia coli* overexpressing CAMP factor-vaccinated mice. (**a**) Sera from individuals without acne (normal control) and acne patients (acne) were assayed by ELISA for antibody to CAMP factor. Antibody (IgG) titers of were determined by using recombinant CAMP factor or GFP as a capture antigen for coating onto a 96-well ELISA plate. The endpoint was defined as the dilution of serum on CAMP factor-coated wells producing the same $OD_{570-450}$ as a 1/100 dilution of serum on GFP-coated wells. Sera negative at the lowest dilution tested were assigned endpoint titers of 100. The data were presented as geometric mean endpoint ELISA titers. (**b**) For reduction of cytotoxicity of *P. acnes*, RAW264.7 macrophage cells were incubated with *P. acnes* ATCC 6919 along with different titers (as indicated) of inactivated sera obtained from individuals without acne, acne patients, or mice vaccinated with *E. coli* overexpressing CAMP factor or GFP for 18 hours. The cytotoxicity of *P. acnes* was determined by an ACP assay and calculated as the percentage of cell death. **P* < 0.05, ***P* < 0.01 by Student *t* test. ACP, acid phosphatase; CAMP, Christie-Atkins-Munch-Petersen; GFP, green fluorescent protein; OD, optical density.

1:1,562,500, respectively (Figure 3a). In the presence of aluminum, vaccination of 10 or 20 μg CAMP factor versus green fluorescent protein conferred effective protection against *P. acnes* in terms of suppression of the production of the MIP-2 cytokine and colonization of *P. acnes* in ear skin (see Supplementary Figure S1 online). These results clearly show that vaccination with *P. acnes* CAMP factor elicited protective immunity against *P. acnes*.

The presence of *P. acnes* CAMP factor in acne lesions

The immunity in mouse ears may not completely recapitulate that in human acne lesions. Human acne explants provide the closest laboratory model attainable to the in vivo environment in terms of local immune response and fidelity to human physiology (Ng et al., 2009). Punch biopsy samples of nonlesional and lesional (2−14 days after onset of inflammatory papules) back skin of patients with acne vulgaris were obtained (see Supplementary Figure S2 online). Acne lesions were used to establish the ex vivo acne explants, which develop open lesions with thickened and inflamed epidermal layers (Figure 4a−c). We first examined whether CAMP factor is present within acne lesions. After homogenization, total cellular RNA was extracted for reverse transcription quantitative PCR analysis (Figure 4d), and the supernatants were used for ELISA assay (Figure 4e). Both the mRNA and protein expressions of *P. acnes* CAMP factor in acne lesions were substantially higher than those in nonlesional skin. Immunohistochemical staining using a monoclonal antibody (mAb) to the *P. acnes* CAMP factor was performed to determine the distribution of CAMP factor in nonlesional and acne lesional skin. The CAMP factor can be detected in hair follicles and sebaceous glands in nonlesional skin (Figure 4g−j), whereas it was distributed everywhere in an acne lesion (Figure 4l−o).

Evaluation of efficacy of CAMP factor mAb in ex vivo acne models

To measure the expression of proinflammatory cytokines in acne vulgaris, 11 cytokines related to T helper (Th) type 1 (IFN-γ, tumor necrosis factor-β, and IL-8 [a human counterpart of murine MIP-2]), Th2 (IL-4, IL-9, IL-10, and IL-13), and Th17 (IL-1α, IL-1β, IL-17A, and transforming growth factor [TGF]-β1) were quantified in nonlesional and acne lesional skin in acne patients. As shown in Figure 5a, the mRNA expression levels of eight cytokines (IFN-γ, tumor necrosis factor-β, IL-8, IL-4, IL-10, IL-1α, IL-1β, and IL-17A) in acne lesional skin were significantly higher than those in nonlesional skin. The mRNA expressions of IL-8 and IL-1β normalized to those of GAPDH were extremely high in acne lesional skin. The protein levels of IL-8 (4,474.0 ± 901.9 pg/mg) and IL-1β (361.2 ± 93.0 pg/mg) in acne lesional skin were noticeably higher than those in nonlesional skin in acne patients and normal skin in healthy individuals (Figure 5b and c).

Incubation of acne ex vivo explants with the mAb to CAMP factor permits the mAb direct access to secreted CAMP factor in acne lesions. The method also allows us to quantify the proinflammatory cytokines released from immune or skin cells in ex vivo explants, predicting the feasibility of immunotherapy using CAMP factor vaccination for the treatment of

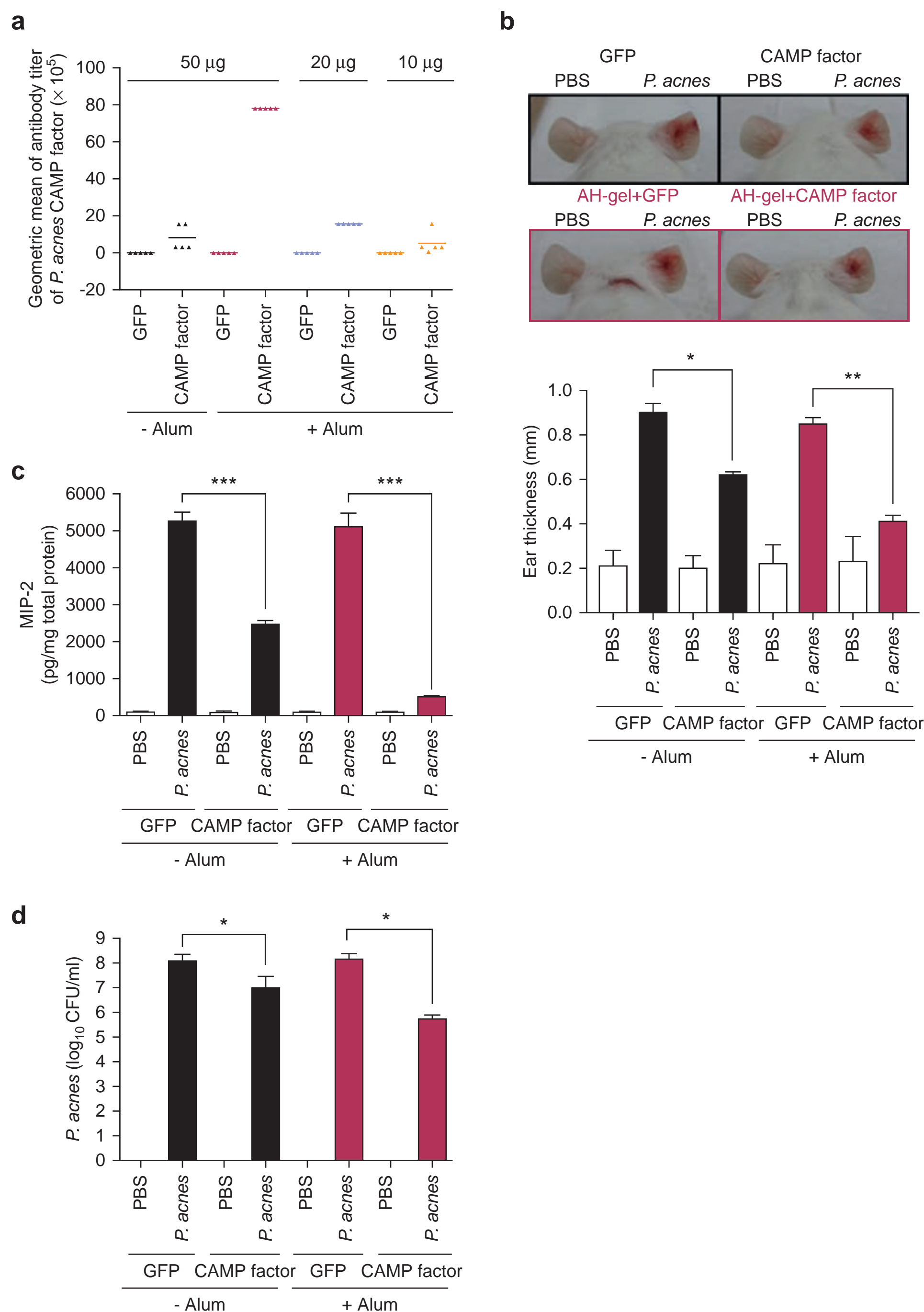

Figure 3. The protective immunity in mice vaccinated with recombinant CAMP factor. Institute of Cancer Research mice were subcutaneously vaccinated with 10, 20, or 50 μg recombinant CAMP factor or GFP with or without 2% aluminum (alum) adjuvant in a 2-week interval. (**a**) Three weeks after vaccination, the titer of antibodies to CAMP factor was measured by ELISA. (**b**) *Propionibacterium acnes* (10^7 CFU/20 μl in PBS) bacteria or PBS (20 μl) was intradermally injected into the right or left ears of GFP- or CAMP factor-vaccinated mice, respectively, 3 weeks after the second booster. Ear thickness (mm) was measured 1 day after bacterial injection. (**c**) The levels of proinflammatory MIP-2 cytokines in the homogenates of *P. acnes*- or PBS-injected ears were measured by ELISA. (**d**) Bacterial colonization (CFUs) in the ears injected with *P. acnes* or PBS were enumerated by plating serial dilutions ($1:10^1$–$1:10^5$) of ear homogenate on an agar plate. Three separate experiments with five mice per group were performed. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student *t* test. Alum, aluminum; CAMP, Christie-Atkins-Munch-Petersen; CFU, colony forming unit; GFP, green fluorescent protein; PBS, phosphate buffered saline.

acne vulgaris in humans and confirming the role of *P. acnes* CAMP factor in the pathogenesis of acne vulgaris.

In a Western blot analysis, we found that the mAb to CAMP factor specifically recognized *P. acnes* CAMP factor 2 (data not shown). Punch biopsy samples of nonlesional and lesional skin from the backs of patients with acne vulgaris were collected as shown in Figures 4a–e. To establish the ex vivo skin explants (Ng et al., 2009), punch skin biopsy samples were fully covered by antibiotic-free media with the epidermal layer side facing up. The ex vivo explants derived from acne lesional and nonlesional skin were incubated with the mAb to CAMP factor for 24 hours. Incubation of ex vivo explants with the mAb to hepatitis B surface antigen served as a control. As shown in Figure 5d and e , the mAb to CAMP factor exhibited the neutralizing capability to attenuate the production of both IL-8 and IL-1β in ex vivo explants obtained from acne lesional skins. Because sebocytes, key cells in a pilosebaceous unit, express high levels of IL-6, we measured IL-6 levels in ex vivo explants and a three-dimensional culture of SZ95 sebocyte cells. As shown in Supplementary Figures 3 and 4 online, the mAb to CAMP factor efficiently reduced the levels of IL-6 in ex vivo acne explants and *P. acnes*-induced IL-6 secretions from sebocyte grown in a three-dimensional culture. These results validate

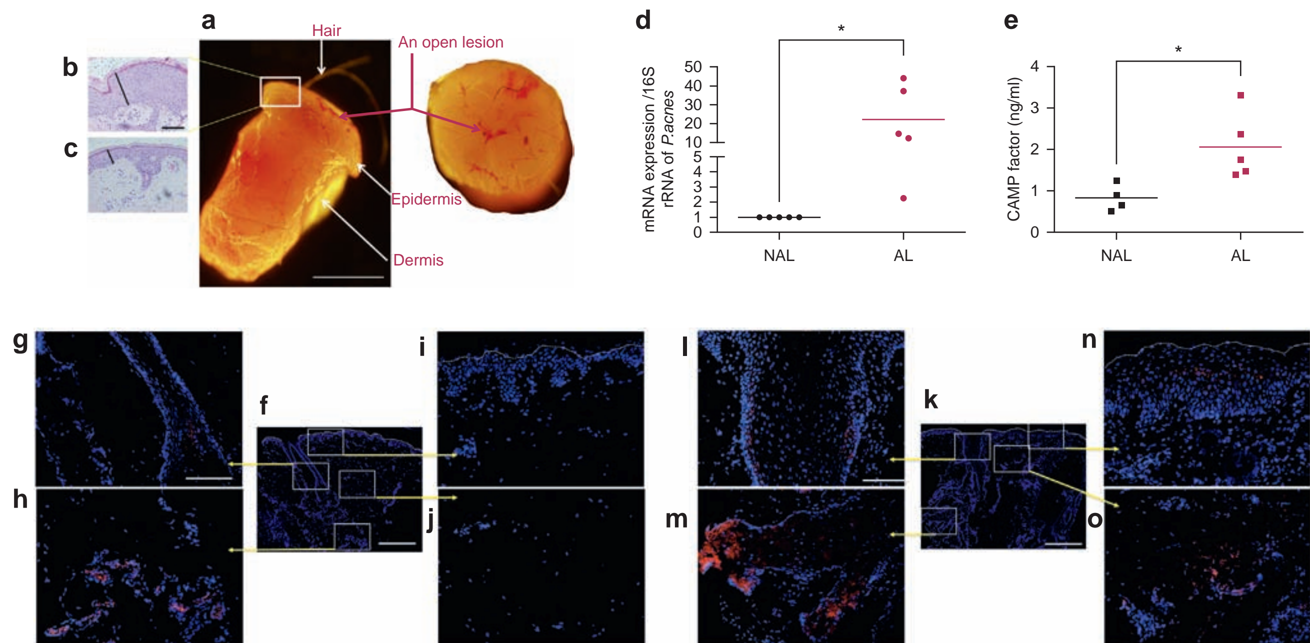

Figure 4. The presence of *Propionibacterium acnes* CAMP factor in acne lesions. (**a**) Images of the biopsy sample (4 × 4 × 8 mm) of lesional back skin of patients with acne vulgaris within 2–14 days of onset of inflammatory papules. An open acne lesion, a hair shaft, and epidermal and dermal layers were indicated. The lesion was illustrated in a top-view picture of an acne biopsy sample with the epidermis facing up. Scale bar = 4 mm. Hematoxylin and eosin staining showed that the epidermis (**b**) in acne lesional skin was thicker than that (**c**) in nonlesional skin. Scale bar = 4 mm. The mRNA expression normalized to (**d**) 16S rRNA of *P. acnes* and (**e**) protein levels of CAMP factor detected by reverse transcription quantitative PCR and ELISA, respectively, in acne lesional skin (AL) were higher than those in nonlesional skin (NAL). $^*P < 0.05$ by Student *t* test. High-magnification photos of selected areas in (**f**) nonlesional skin and (**k**) acne lesional skin are shown in **g**–**j** and **l**–**o**, respectively. (**g, l**) CAMP factor (red stains) was detectable in the hair follicle and was expressed at a higher abundance in (**h, m**) sebaceous glands, (**i, n**) epidermis, and (**j, o**) dermis in acne lesional skin than in nonlesional skin. Scale bars = 500 μm in **f** and **k** and 100 μm in **g** and **l**. CAMP, Christie-Atkins-Munch-Petersen.

the efficacy of the mAb to *P. acnes* CAMP factor in the suppression of inflammation in acne vulgaris.

DISCUSSION

Although *P. acnes* CAMP factor triggered cell death (Huang et al., 2008), it has been described that cell death is a key factor that induces the proinflammatory and anti-inflammatory responses in bacterial infection (Pinheiro da Silva and Nizet, 2009). The CAMP factor acts as a pore-forming toxin that may induce cytolysis and cytokine secretion via activation of the inflammasome (Averette et al., 2009). Toxin-induced membrane permeability results in a decrease in cytoplasmic potassium, which triggers the formation of the inflammasome, leading to caspase-1 activation (Averette et al., 2009). These findings indicate that inflammation and cytolysis occur together in infected tissues. The granulomatous inflammation induced by *P. acnes* (Figures 1c and 3b) may result from a lesion of epithelioid macrophages surrounded by a lymphocyte cuff (Carpinteiro et al., 2008).

Although ex vivo skin explants were used for incubation of mAb for 24 hours the same day they were collected (Figure 5), it has been documented that skin explants exhibit rapid sebaceous gland degradation and disruption of epidermal and dermal integrity after 6 days of ex vivo maintenance (Nikolakis et al., 2015). Besides a three-dimensional culture of sebocyte cells (see Supplementary Figure S4) with a sebaceous-like phenotype (Barrault et al., 2012), other skin models with insertion of complete pilosebaceous unit (Michel et al., 1999) will be used to examine the efficacy of mAb to CAMP factor for suppression of *P. acnes*-induced secretion of proinflammatory cytokines. CAMP factor is a protein named by its biological function of CAMP reaction. The sequence homology of *P. acnes* CAMP factor to other CAMP factors among different bacteria is low. It has been reported that *P. acnes* types IA, IB, or II bacteria encode five different CAMP factor (CAMP factors 1–5) genes (Valanne et al., 2005). The CAMP factor 2 of *P. acnes* in this study shares approximately 36% to 49% amino acid sequence identity with other CAMP factor homologues in *P. acnes*. Previous studies via proteomics have shown that CAMP factor 2 is secreted from all five human-isolated *P. acnes* strains (Sorensen et al., 2010). Only CAMP factor 2 and 4 are detectable in the secretion of the *P. acnes* (KPA171202) strain (Sorensen et al., 2010). Also, it has been shown that CAMP factor 2, but not 4, is the major active co-hemolytic factor of *P. acnes* (Sorensen et al., 2010). Here, we conclude that CAMP factor 2 is essential for *P. acnes*-induced inflammation because mutation of CAMP factor 2 in *P. acnes* (Figure 1c–f) abolished the inflammatory response caused by *P. acnes*. Antibodies produced by CAMP factor-vaccinated mice target secretory CAMP factor instead of bacterial particles. As shown in Figure 1e and f, the bacterial load in the mouse ear was significantly reduced, implying that that CAMP factor vaccination may disarm bacteria that could be eliminated locally and naturally by host immune systems. *P. acnes* CAMP factor was differentially expressed in distinct types of inflammatory acne vulgaris (Quanico et al.,

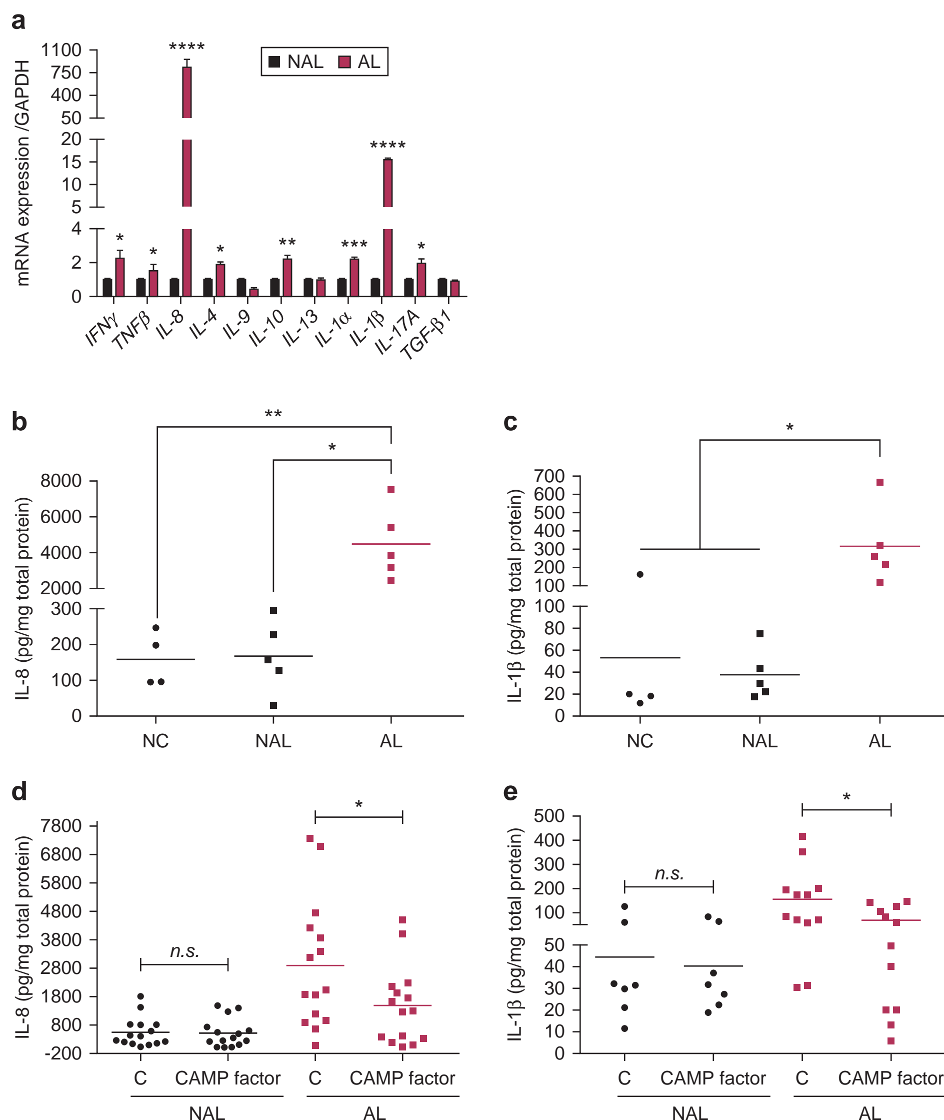

Figure 5. Reduction of *Propionibacterium acnes*-induced inflammation by monoclonal antibody to CAMP factor. Human skin was obtained by taking 4-mm punch biopsy samples from healthy individuals (NC), nonlesional skin (NAL), and acne lesional skin in patients with acne vulgaris(AL). (**a**) The mRNA expressions of 11 Th1/Th2/Th17-related proinflammatory cytokines normalized to GAPDH in nonlesional and acne lesional skin were examined by reverse transcription quantitative PCR. (**b, c**) The protein levels of IL-8 and IL-1β in the skin of healthy individuals and nonlesional and acne lesional skin of acne patients were measured by ELISA. (**d, e**) The nonlesional and acne lesional skin were cut in half, and half was incubated with monoclonal antibody (IgG1) to *P. acnes* CAMP factor for 24 hours in antibiotic-free the EpiLife keratinocyte medium (Thermo Fisher Scientific, Waltham, MA). The other half was incubated with HBsAg monoclonal antibody as a control (C). The protein levels of IL-8 and IL-1β in skins after monoclonal antibody incubation were quantified by ELISA. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ by Student *t* test. CAMP, Christie-Atkins-Munch-Petersen; HBsAg, hepatitis B surface antigen; n.s., not significant; TGF, transforming growth factor; Th, T helper.

2017). There is no conclusive evidence showing the correlation of CAMP factor expression with acne severity and the role of CAMP factor in the pathogenesis of acne vulgaris. Future studies will include (i) examining whether CAMP factor 2-targeted vaccines can diminish the inflammation caused by various *P. acnes* subtypes, (ii) determining if antibodies generated by CAMP factor 2 vaccination are cross-reactive with other CAMP factor homologues, and (iii) creating a multivalent vaccine composed of a mixture of various recombinant CAMP factor homologues because vaccination of mice with CAMP factor 2 does not fully suppress *P. acnes*-induced inflammation (Figure 3c).

The antibodies to CAMP factor are detectable, although low, in human blood (Figure 2a). It has been shown that pre-existing antibodies, acting in concert with complement, are endogenous adjuvants for the generation of protective $CD8^+$ T cells after vaccination against visceral leishmaniasis (Stager et al., 2003). The pre-existing antibodies to respiratory syncytial virus are present in low titers in human sera and are a short-lived antibody (Welliver et al., 1980). Because of relatively rapid decay of the pre-existing antibodies after respiratory syncytial virus infection, respiratory syncytial virus vaccines may be best given more frequently before the expected exposure. Because vaccines that contain toxic antigens are less acceptable candidates for human application, future work will need to include engineering a nontoxic yet highly immunogenic vaccine by either a chemical or genetic approach (Sheng and Kong, 2012) to achieve clinical utility.

Although aluminum is a known Th2 adjuvant, it has been shown to potentiate either the Th1 or Th2 immune response (Averette et al., 2009). Thus, it is worthwhile to determine which T-cell populations (Th1, Th2, and/or Th17) are activated by vaccination with *P. acnes* CAMP factor in the presence or absence of aluminum. *P. acnes* bacteria stimulate skin cells to produce cytokines, leading to inflammatory acne disease (Contassot and French, 2014; Kurokawa et al., 2009; Nagy et al., 2006; Nakatsuji et al., 2011; Qin et al., 2014). IL-17, expressed in acne lesions (Agak et al., 2014), can be induced by *P. acnes* bacteria in peripheral blood mononuclear cells. Recently, Th1- (IFN-γ and IL-8), Th2- (IL-10), and Th17- (IL-1β and TGF-β) related cytokines were

strongly up-regulated in acne lesions (Kelhala et al., 2014). This evidence supports our findings in Figure 5a–c showing that the levels of IL-8 and IL-1β mRNA and protein expression in acne lesions were higher than those in nonlesional skin. *P. acnes* bacteria play a key role in the development of inflammatory lesions via induction of secretion of IL-6 and IL-8 by follicular keratinocytes, IL-1β, tumor necrosis factor-α, IL-8, and IL-12 by monocytic cells in a toll-like receptor 2–dependent manner (Bojar and Holland, 2004; Kurokawa et al., 2009; Leeming et al., 1988; Lheure et al., 2016; Vowels et al., 1995) and IL-1β, IL-12, and IL-23 by peripheral blood mononuclear cells (Ng et al., 2009). In addition, sebocytes secreted IL-8 in response to *P. acnes* exposure (Nagy et al., 2006). *P. acnes* bacteria triggered mixed Th17/Th1 responses by inducing the simultaneous secretion of IL-17A and IFN-γ from specific $CD4^+$ T cells (Agak et al., 2014; Kistowska et al., 2015). As shown in Figure 5a, the mRNA expressions of eight cytokines were significantly higher in acne lesions compared with those in nonlesional skin in acne patients. Our results are in line with the prior observations showing up-regulated IFN-γ, IL-8, IL-10, IL-1β, and IL-17A in acne lesions examined by reverse transcription quantitative PCR (Bechara et al., 2012; Kang et al., 2005; Kelhala et al., 2014; Kelhala et al., 2016). However, our results showed no significant difference in the mRNA expression of TGF-β1 between acne lesional and nonlesional skin. TGF-β1 is a well-studied player in the pathogenesis of scarring (Klass et al., 2009), an event in the late stages of acne vulgaris. It has been reported that overproduction of TGF-β1 can result in excessive deposition of scar tissue and fibrosis (Ignotz and Massague, 1986). The acne lesions in this study were isolated from lesions within 2–14 days of onset of inflammatory papules, not in the late stages. This may explain why no difference in TGF-β1 expression between acne lesional and nonlesional skin was noted.

Although effective in decreasing expression of TGF-β1 in skin cells (Carroll et al., 2002; Furuzawa-Carballeda et al., 2005), triamcinolone acetonide has known adverse effects for intralesional injection (Levine and Rasmussen, 1983). If the humanized mAb to CAMP factor can be developed, the injection of the mAb to CAMP factor directly into acne lesions potentially can replace triamcinolone acetonide for intralesional therapy against acne vulgaris. The vaccination approaches developed in this study may provide an effective means with protective immunity against acne vulgaris. The vaccination may benefit not only patients with acne vulgaris but also those with other *P. acnes*-associated diseases including prostate cancers, polymer (device)-associated diseases, sepsis, toxic shock syndrome, endocarditis, osteomyelitis, and various surgery infections (Fassi Fehri et al., 2011; Haidar et al., 2010; Perry and Lambert, 2011; Severi et al., 2010).

MATERIALS AND METHODS

Ethics

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and an approved Institutional Animal Care and Use Committee protocol (no. S10058) at University of California, San Diego. The institutional review board at UCSD approved the procedure of written informed patient consent and sampling of acne biopsy samples under an approved protocol (no. 121230).

Bacterial culture

P. acnes bacteria including ATCC 6919, wild-type *P. acnes* (266; 1-1a, ST18) strain (Bruggemann et al., 2004), and a knock-out mutant of CAMP factor 2 (*Δcamp2*, [PPA0687]) (Nakatsuji et al., 2008b; Sorensen et al., 2010) were used.

Mass spectrometry, vaccination, in vitro neutralization, and ex vivo acne model

All procedures for the identification of *P. acnes* CAMP factor by isotope-coded protein labeling (Schmidt et al., 2005) using light ($^{12}C_6$) and heavy ($^{13}C_6$) forms of *N*-nicotinoyloxy-succinimide for liquid chromatography linear ion trap quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA), the expression of recombinant CAMP factor, vaccination, quantification of antibody titers, injection of *P. acnes* into mouse ear, in vitro neutralization, ELISA, bacteria counts, and establishment of ex vivo acne model were performed according to the methods described in Nakatsuji et al. (2008a) and are described in detail in the Supplementary Materials online.

CONFLICT OF INTEREST

CCZ is owner of an international patent of the human immortalized sebaceous gland cell line SZ95 (WO0046353). The other authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

CMH conceived the project, designed experiments, supervised the research, and assisted in analyzing data and writing the manuscript. YW performed the experiments and prepared the figures. TRH and YLT wrote the institutional review board protocol, assisted in writing the manuscript, and collected skin biopsy samples. CCZ provided the SZ95 sebocyte cell lines and protocols for cell culture. RLG provided clinical knowledge and designed experiments.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.05.032.

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