Staphylococcus aureus Hijacks a Skin Commensal to Intensify Its Virulence: Immunization Targeting β-Hemolysin and CAMP Factor

Chih-Wei Lo[1,2,3], **Yiu-Kay Lai**[3], **Yu-Tsueng Liu**[4,5], **Richard L. Gallo**[1,2], and **Chun-Ming Huang**[1,2,5]

[1]Division of Dermatology, Department of Medicine, University of California, San Diego, San Diego, California, USA

[2]VA San Diego Healthcare Center, San Diego, California, USA

[3]Department of Life Science, Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan

[4]Division of Infectious Diseases, Department of Medicine, University of California, San Diego, San Diego, California, USA

[5]Moores Cancer Center, University of California, San Diego, San Diego, California, USA

Abstract

The need for a new anti-*Staphylococcus aureus* therapy that can effectively cripple bacterial infection, neutralize secretory virulence factors, and lower the risk of creating bacterial resistance is undisputed. Here, we propose what is, to our knowledge, a previously unreported infectious mechanism by which *S. aureus* may commandeer *Propionibacterium acnes*, a key member of the human skin microbiome, to spread its invasion and highlight two secretory virulence factors (*S. aureus* β-hemolysin and *P. acnes* CAMP (Christie, Atkins, Munch-Peterson) factor) as potential molecular targets for immunotherapy against *S. aureus* infection. Our data demonstrate that the hemolysis and cytolysis by *S. aureus* were noticeably augmented when *S. aureus* was grown with *P. acnes*. The augmentation was significantly abrogated when the *P. acnes* CAMP factor was neutralized or β-hemolysin of *S. aureus* was mutated. In addition, the hemolysis and cytolysis of recombinant β-hemolysin were markedly enhanced by recombinant CAMP factor. Furthermore, *P. acnes* exacerbated *S. aureus*-induced skin lesions *in vivo*. The combination of CAMP factor neutralization and β-hemolysin immunization cooperatively suppressed the skin lesions caused by coinfection of *P. acnes* and *S. aureus*. These observations suggest a previously unreported immunotherapy targeting the interaction of *S. aureus* with a skin commensal.

Correspondence: Chun-Ming Huang, Division of Dermatology, Department of Medicine, University of California, San Diego, Room 276, 3525 John Hopkins Court, San Diego, California 92121, USA. chunming@ucsd.edu or Yu-Tsueng Liu, Moores Cancer Center, University of California, San Diego, 3855 Health Sciences Drive, La Jolla, California 92093-0815, USA. ytliu@ucsd.edu.

CONFLICT OF INTEREST
The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

INTRODUCTION

Staphylococcus aureus, a Gram-positive bacterium, is a major causes of fatal nosocomial infections (Homa and Palfreyman, 2000). It is estimated that *S. aureus* accounts for 12 million outpatient visits and 292,000 hospitalizations annually in the United States alone, of which 126,000 are due to methicillin-resistant *S. aureus* (MRSA) (Goetghebeur *et al*., 2007). Recent studies estimated that more people die from MRSA bacterium than from HIV in the United States (Klevens *et al*., 2008; Payne, 2008). In addition, MRSA infections have recently been found in increasing numbers among individuals in the community without health-care exposure (Wilson and Rinker, 2009). Although *S. aureus,* including MRSA and community-associated MRSA, can cause life-threatening and systemic infection, skin and soft tissue are the most common sites of *S. aureus* infection, comprising > 75% of MRSA disease (Cohen *et al*., 2007).

Nearly everyone hosts *Propionibacterium acnes* (Brook and Frazier, 1991; Ahn *et al*., 1996), which accounts for approximately half of the total skin microbiome (Tancrede, 1992), with an estimated density of 10^2–10^{5-6}cm^2 (McGinley *et al*., 1978; Leyden *et al*., 1998). *P. acnes* predominates (> 60% of total bacteria) in facial skin (Grice *et al*., 2009); however, it can be found almost everywhere on the body (Yamada *et al*., 2002; Nishiwaki *et al*., 2004). There is growing evidence that *P. acnes* and *S. aureus* coexist in many human diseases, including acne lesions (Williams *et al*., 1992), implant infections (Ramage *et al*., 2003; Bashir *et al*., 2007), and sepsis (Duncan and Sperling, 2008). This indicates that the pathogenic *S. aureus* may interact with residential *P. acnes* during infection in humans, and it might therefore be important to understand the role of *P. acnes* in *S. aureus* infection.

Developing effective therapeutic approaches for *S. aureus* or MRSA treatment remains an unmet challenge because of its formidable resistance against multiple traditional antibiotics such as methicillin, cloxacillin, and flucloxacillin (Takizawa *et al*., 2005; Takano *et al*., 2007). Current antibiotic therapy nonspecifically eliminates the majority of bacteria, which impacts human microbiome homeostasis (Jeong *et al*., 2009) and increases the risk of developing resistant bacteria. Past efforts to generate vaccines against *S. aureus* have been unsuccessful. Anti–*S. aureus* vaccines targeting surface proteins (e.g., clumping factor) have failed in clinical trials (Shinefield and Black, 2005; DeJonge *et al*., 2007). Other anti–*S. aureus* vaccines targeting surface molecules (e.g., poly-*N*-succinyl β-1 –6-glucosamine (McKenney *et al*., 1999) and iron-regulated surface determinants A, B, D, and E (Stranger-Jones *et al*., 2006)) have subsequently been developed. A recent vaccine using a secretory toxin (α-hemolysin) as an antigen (Bubeck Wardenburg and Schneewind, 2008) emphasizes the notion that targeting secretory virulence factors rather than bacterial surface proteins creates a more effective therapy with a lower tendency to select for resistance. Although these vaccines had been demonstrated to be effective in mice, none of them was constructed taking into account the interaction of *S. aureus* with human commensal bacteria. Without this consideration, the efficacy of these vaccines becomes doubtful when they are translated from mice to humans.

Here, we have demonstrated that the secretory CAMP (Christie, Atkins, Munch-Peterson) factor of *P. acnes* enhances hemolysis and cytolysis by *S. aureus* β-hemolysin, suggesting that *S. aureus* may shrewdly utilize the secreted *P. acnes* CAMP factor to intensify its virulence. The results revealed an intriguing interaction of *S. aureus* with human commensals at the initial stage of infection and two potential therapeutic targets (*P. acnes* CAMP factor and *S. aureus* β-hemolysin) for treatment of *S. aureus* infection.

RESULTS

P. acnes amplified the hemolytic activity of *S aureus*

When *S. aureus* (*S. aureus* 113) was grown with *P. acnes* (ATCC 6919) on a sheep blood agar plate, we found that the hemolytic activity of *S. aureus* was dramatically augmented in comparison with that of *S. aureus* or *P. acnes* alone (Figure 1a). Accordingly, the colony-forming units (CFUs) of *S. aureus* in culture alone or in a coculture of *S. aureus* and *P. acnes* were not distinct (Supplementary Figure S1 online), suggesting that *P. acnes* did not influence the growth of *S. aureus*. However, this augmentation of hemolysis did not appear when *S. aureus* 113 was cocultured with *Staphylococcus epidermidis* (ATCC 12228), one of the human skin commensal bacteria (Figure 1b; Otto, 2009), which indicated that hemolytic augmentation of *S. aureus* was specifically mediated by *P. acnes* and was not a result of an increase in the growth of *S. aureus*.

P. acnes CAMP factor is involved in the hemolysis and cytolysis of *S. aureus*

It has been reported that the CAMP factors of *Streptococcus agalactiae* (Schneewind *et al.*, 1988), *Streptococcus uberis* (Jiang *et al.*, 1996), and *Streptococcus pyogenes* (Gase *et al.*, 1999) exhibit the CAMP reactions that can enhance the hemolytic activity of *S. aureus*. Spotting *P. acnes* CAMP factor (accession number AY726656), but not a control green fluorescence protein (GFP), considerably increased the intensity of the diffusion zone of the hemolysis of *S. aureus* (Figure 2a), supporting the notion that the *P. acnes* CAMP factor with a CAMP reaction exerts cohemolytic activity. It has been known that macrophages are essential for the initiation and execution of inflammatory response and subsequent resolution of bacterial infection (Kobayashi *et al.*, 2006). We exposed murine RAW264.7 macrophage cells to culture supernatants of bacteria and found that secretory components from *P. acnes* and *S. aureus* cooperatively enhance the cytolysis of *S. aureus* (Figure 2b). In addition, neutralization of *P. acnes* CAMP factor markedly suppressed the cytotoxicity of macrophages induced by the supernatants of bacterial coculture (Figure 2b), indicating that CAMP factor was involved in the enhancement of cytolysis of *S. aureus*. The antisera were obtained from mice as described in Materials and Methods. The specificity of antiserum against CAMP factor was verified by western blotting (shown in the inset panel in Figure 2b).

Induction of proinflammatory cytokines also has a crucial role in the progression of bacterial infection. We next examined whether *P. acnes* alters the levels of *S. aureus*–induced proinflammatory cytokines *in vivo*. Ears of imprinting control region (ICR) mice were intradermally injected with bacteria for 24 hours and subsequently excised to homogenize for detection of macrophage inflammatory protein-2 (MIP-2) levels in the supernatants. As shown in Figure 2c, coinjection of *P. acnes* and *S. aureus* significantly elevated the level of MIP-2 (11.87 ± 1.72 ng ml^{-1}) compared with the injection of *P. acnes* (2.58 ± 0.94 ng ml^{-1}) or *S. aureus* (8.78 ± 1.23 ng ml^{-1}) alone. Subsequently, the neutralization of *P. acnes* with anti-CAMP factor (5.76 ± 1.48 ng ml^{-1}), but not anti-GFP (14.61 ± 3.36 ng ml^{-1}), antiserum notably reduced the level of MIP-2 induced by bacterial coinjection. The data in Figure 2 indicate that *P. acnes* CAMP factor exhibited cohemolytic activity and was an indispensable secretory protein in *P. acnes* that enhanced the virulence of *S. aureus*.

S. aureus β-hemolysin is associated with CAMP factor in the enhancement of hemolysis and cytolysis

It has been documented that *P. acnes* CAMP factor can potentiate the hemolytic activity of *S. aureus* β-hemolysin, a secretory virulence factor (Huseby *et al.*, 2007). Spotting recombinant CAMP factor with recombinant β-hemolysin, but not with recombinant α-hemolysin, on a sheep blood agar plate for 1 day showed synergistic enhancement of

hemolysis (Figure 3a). The hemolysis assay showed that incubation of erythrocytes with wild-type *S. aureus* and *P. acnes* displayed greater hemolytic activity than incubation with individual *S. aureus* or *P. acnes* (Figure 3b). The enhancement of hemolytic activity was completely abrogated when *P. acnes* was coincubated with β-hemolysin-deficient (SAhlb⁻) strains (Figure 3b), but not with α-hemolysin (SAhla⁻) strains (Supplementary Figure S2 online). To further investigate whether β-hemolysin strengthened the cytolysis of CAMP factor, RAW264.7 cells were treated with 200 µgml⁻¹ of recombinant GFP, CAMP factor, β-hemolysin, or a mixture of β-hemolysin plus CAMP factor or GFP for 18 hours (Figure 3c). As expected, the cytolysis of β-hemolysin ($37.92 \pm 2.4\%$) was strikingly reinforced when cells were treated with the mixture of β-hemolysin and CAMP factor ($81.09 \pm 5.45\%$).

P. acnes exacerbates *S. aureus*–induced skin lesions

To gain insight into the interaction of *S. aureus* and *P. acnes in vivo*, skin lesions were made by injection of bacteria into the dorsal skin of ICR mice, a mouse strain with diversity in gene function that more closely resembles the human population. Injection of wild-type *S. aureus* (National Collection of Type Cultures (8325-4; $11.11 \pm 3.96mm^2$), but not *P. acnes*, for 2 days induced the development of skin lesions (Figure 4) that recapitulate an ulcerative response of *S. aureus* infection in humans. The lesions ($29.01 \pm 4.75mm^2$) were significantly exacerbated when mice were coinjected with *S. aureus* and *P. acnes*.

S. aureus β-hemolysin is immunogenic

To explore the value of a therapy targeting β-hemolysin, we first examined the antigenicity of β-hemolysin. An expressed protein matching β-hemolysin with a molecular weight of ~33 kDa was detected by SDS-PAGE after isopropyl-β-ᴅ-thiogalactopyranoside induction (Figure 5a left panel) and protein purification (Figure 5a right panel). It was further validated via sequencing by Nano liquid chromatography linear trap quadrupole tandem mass spectrometry (West Palm Beach, FL) after in-gel trypsin digestion (Figure 5b). Twenty-seven peptides were fully sequenced and matched well with internal amino acids of *S. aureus* β-hemolysin (Supplementary Table S1 online). A sequenced peptide (NNDVVIFNEAFDNGASDK; 78–95 amino acid residues) of β-hemolysin is presented in Figure 5b. To examine the immunogenicity of β-hemolysin, we immunized ICR mice intranasally with UV-inactivated *E. coli* overexpressing β-hemolysin or GFP (a control protein). Immunization of UV-inactivated *E. coli* overexpressing antigens has been demonstrated in our previous efforts (Liu *et al.*, 2008). A single band was detected in western blot analysis when recombinant β-hemolysin was immunoreacted with serum obtained from immunized mice, indicating that β-hemolysin was immunogenic (Figure 5c).

The combination of β-hemolysin immunization with passive neutralization of CAMP factor confers protection against skin lesions

To assess the effect of immunization with UV-inactivated *E. coli* overexpressing β-hemolysin and/or neutralization of CAMP factor on the bacteria-induced skin lesions, immunized mice were injected subcutaneously with both *S. aureus* and *P. acnes* in the presence of antiserum against CAMP factor or GFP. In GFP-immunized mice, a massive lesion size ($30.01 \pm 12.96mm^2$) developed after injection with bacteria in the presence of antiserum against GFP. However, the lesion ($7.91 \pm 2.67mm^2$) was significantly decreased in β-hemolysin-immunized mice (Figure 5d). Similarly, the lesion ($9.53 \pm 3.83mm^2$) was markedly attenuated in GFP-immunized mice accompanied by passive neutralization of antiserum against CAMP factor (Figure 5d and e). The results indicated that immunization targeting either *S. aureus* β-hemolysin or *P. acnes* CAMP factor can effectively protect mice from skin lesions caused by coinfection of *S. aureus* and *P. acnes*. To exclude the possibility of anti-CAMP factor antibody crossreacting with *S. aureus* directly, the *S. aureus* bacteria treated with and without anti-CAMP factor antiserum were subcutaneously injected into the

dorsal skin of ICR mice to induce skin lesions. As shown in Supplementary Figure S3 online, there is no difference in the size of lesions caused by *S. aureus* treated with or without anti-CAMP factor antiserum, suggesting that anti-CAMP factor antiserum did not affect the *S. aureus*-induced skin lesions.

To investigate whether abrogation of both β-hemolysin and CAMP factor provided greater protection, mice immunized to β-hemolysin accompanied by passive neutralization of antiserum against CAMP factor were coinjected with *S. aureus* and *P. acnes*.The bacteria-induced lesion size ($3.46 \pm 1.17 \mathrm{mm}^2$) was considerably reduced only in the case of both *S. aureus* β-hemolysin immunization and *P. acnes* CAMP factor neutralization (Figure 5d and e). The result clearly illustrated that the combination of β-hemolysin immunization and passive neutralization of CAMP factor cooperatively suppressed skin lesions caused by co-infection of *S. aureus* and *P. acnes*.

DISCUSSION

In the human microbiome, the full spectrum of microbial species residing in humans, *P. acnes* is ubiquitous in the general population, whereas *S. aureus* is present in ~25% (Miller *et al*., 2007). It has been reported that pathogenic strains of MRSA bacteria have acquired genes from human commensal organisms that are present on the skin (Diep *et al*., 2006; Grice *et al*., 2009). Our data demonstrate that *P. acnes* enhances the hemolysis of *S. aureus* (Figure 1), suggesting that a specific interaction of the bacteria occurs in the human microbiome (Cogen *et al*., 2008; Young and Schmidt, 2008). We identified two secretory toxins—*P. acnes* CAMP factor and *S. aureus* β-hemolysin—that were critically engaged in the interaction of *P. acnes* and *S. aureus*. The hemolytic capacity is thought to be a virulence factor for numerous microbial pathogens to degrade tissue, invade host cells, disseminate themselves, and resist the host immune system. Thus, evidence of the enhancement of hemolysis of *S. aureus* by *P. acnes* raises the possibility that *S. aureus* benefits from *P. acnes* present on the skin to invade its human host.

Staphylococcal hemolysins are recognized as chief virulence factors that contribute to bacterial invasion and escape from the host immune response (Dinges *et al*., 2000). The β-hemolysin, also called sphingomyelinase, is highly active against sheep and bovine erythrocytes (Larsen *et al*., 2002). We observed an augmentation of hemolysis and cytolysis when cells were treated with both *S. aureus* β-hemolysin and *P. acnes* CAMP factor (Figure 3a and c), suggesting that *S. aureus* β-hemolysin may commandeer *P. acnes* CAMP factor to enhance the production of host ceramide for spreading infection in a cell-to-cell manner.

It has been documented that skin and soft tissue infections make up most of the cases of MRSA infections (Miller *et al*., 2005), although community-associated MRSA infections may cause more severe necrotizing pneumonia (Hageman *et al*., 2006) and bacteremia (Seybold *et al*., 2006). Skin can be the first place through which the *S. aureus*/MRSA bacterium enters a human host. Our data illustrated that *P. acnes* enhanced the *S. aureus*–induced skin lesions (Figure 4). Why passive neutralization of *P. acnes* CAMP factor significantly attenuated the skin lesions caused by the coinfection of *S. aureus* and *P. acnes* (Figure 5d and e) has two possible explanations. First, antiserum to *P. acnes* CAMP factor may exhibit cross-reactivity to *S. aureus*, leading to a decrease in skin lesions caused by *S. aureus*. Second, *P. acnes* CAMP factor may be massively secreted while *S. aureus* is present and thus exacerbate existing lesions. Our data (Supplementary Figure S3 online) suggest that anti-CAMP factor antiserum did not affect the skin lesions induced by *S. aureus* alone. However, we cannot rule out the second possibility that *S. aureus* stimulates secretion of *P. acnes* CAMP factor. Given that recombinant CAMP factor can potentiate the hemolytic activity of *S. aureus* (Figure 2a), it is possible that *P. acnes* does not need to be alive to exert

this cohemolytic effect. Mixing killed *P. acnes* with *S. aureus* in future studies will determine whether CAMP factor secretion is necessary to enhance the virulence of *S. aureus*. Direct measurement of β-hemolysin and CAMP factor produced by these bacteria would be useful to determine whether an increase in the production and/or activities of virulence factors leads to the augmentation of cytolysis. In fact, it is worthwhile to clarify a signaling relationship among *S. aureus* β-hemolysin, *P. acnes* CAMP factor, and host ceramide. We have shown that humoral immunity against *P. acnes* CAMP factor can mitigate the *in vitro* hemolytic activity of *S. aureus* cocultured with *P. acnes*. Although we have shown protection against *S. aureus* infected mice through vaccination against β-hemolysin, we do not have enough evidence to conclude whether this is a T or B cell mediated immune response. Adoptive transfer experiments with sera and/or T cells are crucial to understanding the underlying mechanisms.

Although the overgrowth of *P. acnes* can cause many human diseases such as acne vulgaris and toxic shock syndrome (Yang *et al.*, 2009), the bacterium is a major commensal in humans (Grice *et al.*, 2009). Unlike active immunization, passive immunotherapy with local attenuation of CAMP factor will be less disruptive to *P. acnes* in distant locales. The genome of *P. acnes* has five homologs of the cohemolytic CAMP factor of *S. agalactiae* (Jurgens *et al.*, 1987; Lang and Palmer, 2003; Valanne *et al.*, 2005). Here, we have shown that neutralization of one (accession number AY726656) of the CAMP factors in *P. acnes* dramatically suppressed the coinfection-induced skin lesions (Figure 5d and e). Currently, we are determining whether other CAMP factors can also intensify the β-hemolysin toxicity and if they can be neutralized by their respective antisera. In conclusion, we have shown the potential benefit of targeting *P. acnes* CAMP factor for *S. aureus* infections using an animal model. Although disarming bacteria via neutralization of their secretory toxins may enhance the clearance of bacteria by immune cells, the immune-mediated blockade of CAMP factor and/or β-hemolysin in combination with small-molecule inhibitors directly targeting bacterial particles may be of value for complete eradication of bacterial infection.

Overall, the study provides the clinical relevance that passive administration of anti-CAMP factor antibody into *S. aureus* β-hemolysin-immunized patients may (i) prevent systemic bacterial invasion after local colonization at the initial site of infection; (ii) faciliate the host immune system's eradication of disarmed/avirulent bacteria before antibody reaches the infected site; and (iii) instantly rescue the inefficiency of vaccines that provoke low titer of antibody in immunocompromised people or vaccines that have no activity against new strains of *S. aureus*/MRSA.

MATERIALS AND METHODS

Bacterial culture

P. acnes (ATCC 6919; Manassas, VA) was cultured on Brucella broth agar (BD, Sparks, MD) as described previously (Nakatsuji *et al.*, 2009). Single colonies were inoculated in reinforced Clostridium medium(Oxford, Hampshire, UK) and cultured at 37 °C under anaerobic conditions using Gas-Pak (BD Biosciences, San Jose, CA). *S. aureus* 113 (ATCC 35556) and *S. epidermidis* (ATCC 12228) bacteria were from ATCC. A β-hemolysin-deficient *S. aureus* strain and its wild type (National Collection of Type Cultures 8325-4) were obtained from Pyong Park (Division of Respiratory Diseases, Children's Hospital, Harvard Medical School, Boston, MA). An α-hemolysin-deficient *S. aureus* strain was obtained from Bill Schwan (Department of Microbiology, University of Wisconsin–La Crosse, La Cross, WI). *S. aureus* and *S. epidermidis* were cultured on 3% tryptic soy broth (Sigma-Aldrich, St Louis, MO) agar overnight at 37 °C. Bacteria were suspended in an appropriate amount of phosphate-buffered saline (PBS) for the further experiments.

Hemolytic activity of *S. aureus* enhanced by *P. acnes* or CAMP factor

Sheep blood agar plates were used to determine the cohemolytic activity of *P. acnes* or CAMP factor. *P. acnes* (2×10^7 CFUs), *S. aureus* 113 (2×10^7 CFUs), the mixture of *P. acnes* and *S. aureus* 113 (1:1 ratio with a total of 2×10^7 CFUs), *S. epidermidis* (2×10^7 CFUs), and the mixture of *S. epidermidis* and *S. aureus* 113 (1:1 ratio with a total of 2×10^7 CFUs) were all suspended in 10 µl PBS and then streaked on sheep blood agar plates and incubated for 2 days under anaerobic conditions at 37 °C. For determination of cohemolytic activity of CAMP factor, *S. aureus* 113 (10 µl, 2×10^7 CFUs in PBS) was streaked on the sheep blood agar plate. A total of 10 µg of recombinant CAMP factor or GFP within 5 µl PBS was spotted beside the *S. aureus* streak grown at 37°C for 18 hours.

Molecular cloning, expression, and purification of β-hemolysin and CAMP factor

The bacterial genomic DNA was prepared as described previously (Sohail, 1998). For purposes of constructing a plasmid carrying the *hlb* gene encoding the β-hemolysin (accession number: X61716), the gene was amplified by PCR from wild-type *S. aureus* genomic DNA as a template, using oligonucleotide primers of forward primer, 5′-AA<u>GTCGAC</u>ATGGTGAAAAAAACAAAATCC-3′ (*Sal*I restriction site is underlined), and reverse primer, 5′-AT<u>AAGCTT</u>CTATTTACT ATAGGCTTTGATTGGG-3′ (*Hin*dIII restriction site is underlined). The oligonucleotide primers specific for the *hlb* gene were also used for PCR analysis of genomic DNA of various strains of *S. aureus*. The CAMP factor (accession number: AY726656) was cloned from *P. acnes* genomic DNA, using oligonucleotide primers of forward primer, 5′-AT<u>GTCGA</u>CGTCGAGCCGACGACGACCATC TCG-3′ (*Sal*I restriction site is underlined), and reverse primer, 5′-AT <u>AAGCTT</u>GGCAGCCTTCTTGACATCGGGGGGAG-3′ (*Hin*dIII restriction site is underlined). The amplified PCR products and the In-Fusion Ready pEcoli-Nterm $6 \times$ HN expression plasmid (Clontech, Mountain View, CA) were digested with *Sal*I/*Hin*dIII and purified by agarose gel electrophoresis for ligation. The constructed plasmids were transformed and expressed in *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA). A pEcoli-Nterm-GFP plasmid (Clontech) was used to express the GFP. A 2ml overnight culture of *E. coli* BL21 (DE3) containing expression plasmids of β-hemolysin, CAMP factor, or GFP, was inoculated into 200 ml Luria-Bertani and incubated with isopropyl-β-ᴅ-thiogalactopyranoside (1mᴍ; Sigma-Aldrich) for 4 hours. A column with 2 ml Ni-NTA agarose (Qiagen, Valencia, CA) was used for protein expression and refolding. The purified and refolded protein was dialyzed overnight at 4 °C against 5 liters of $1 \times$ PBS by using Spectra/Por molecular-porous membrane tubing (molecular weight cutoff: 3,500; Spectrum Laboratories, Rancho Dominguez, CA).

Protein identification via mass spectrometry

In-gel digestion with trypsin and protein identification via a Nano liquid chromatography linear trap quadrupole tandem mass spectrometry analysis were performed as described previously (Martin and Clynes, 1993; Shi *et al*., 2007). SEQUEST was searched with a fragment ion mass tolerance of 0.5Da and a parent ion tolerance of 1.0Da.

Vaccination and detection of antibodies to β-hemolysin and CAMP factor

Female ICR mice (Harlan, Indianapolis, IN) ~2 months old were used for vaccination. The mice were intranasally vaccinated with *E. coli* BL21 (DE3) overexpressing β-hemolysin, CAMP factor, or GFP (as the control), which were inactivated by UV ($3,500$ Jm^{-2}) irradiation. The inactivation of irradiated *E. coli* BL21 (DE3) was indicated by its inability to grow on a Luria-Bertani agar plate (data not shown). For intranasal vaccination, 25 µl of irradiated (1×10^9 CFUs) *E. coli* BL21 (DE3) overexpressing β-hemolysin, CAMP factor,

or GFP was pipetted into the nasal cavities of mice. ICR mice were vaccinated at 3-week intervals for 9 weeks. One week after the third boost, antisera against β-hemolysin or CAMP factor were collected individually for western blot analysis using a goat anti-mouse IgG (H + L) conjugated to horseradish peroxidase (Promega, Madison, WI) and the ECL Western Blotting Substrate (Pierce, Rockford, IL) for the detection of chemiluminescence.

Proinflammatory MIP-2 detection

The proinflammatory MIP-2 cytokine was determined by sandwich ELISA using a Quantikine M mouse MIP-2 set (R&D Systems, Minneapolis, MN).

Skin infection of *S. aureus*

The wild-type *S. aureus* (1×10^7 CFUs), *P. acnes* (1×10^7 CFUs), and *P. acnes* plus wild-type *S. aureus* (1:1 ratio with a total of 2×10^7 CFUs) were suspended in 50 µl PBS and subsequently injected subcutaneously into dorsal skin of the flank of 8- to 12-week-old female ICR mice, respectively. After injection for 2 days, the lesion sizes were examined and quantified by using ImageJ software (National Institutes of Health, Bethesda, MD).

Protective effect of a β-hemolysin-based vaccine along with CAMP factor neutralization

P. acnes (1×10^7 CFUs in 25µl PBS) was preincubated with 5% (v/v) anti-CAMP factor or anti-GFP antiserum (as the control) at 25°C for 1 hour and then mixed with wild-type *S. aureus* (1×10^7 CFUs in 25µl PBS). Subsequently, the mixtures were subcutaneously injected into the dorsal skin of ICR mice that were vaccinated with irradiated *E. coli* BL21 (DE3) overexpressing β-hemolysin or GFP, respectively. After injection for 2 days, the lesions were measured using ImageJ software.

Hemolysis assay

The defibrinated sheep blood cells (Lampire Biological Laboratories, Pipersville, PA) were incubated with individual bacteria (*P. acnes* or *S. aureus*; 2×10^7 CFUs) or a bacterial coculture (*P. acnes* with various strains of *S. aureus*; 2×10^7 CFUs) suspended in 10µl PBS for 2 days. Incubation of blood cells with Triton X-100 (2%; Sigma-Aldrich) served as a positive control. The samples were then centrifuged at $800g$ for 10 minutes. The absorbance of hemoglobin release was measured at 540 nm and is expressed as percentage of Triton X-100–induced hemolysis. Results given represent mean values from triplicate measurements.

Cell culture and *in vitro* cytotoxicity assay

A murine macrophage cell line, RAW264.7, was cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum at 37°C. Cells (1×10^5) were infected with 1×10^6 CFUs (multiplicity of infection = 1:10) bacteria. For *in vitro* neutralization, complements in sera were deactivated by heating at 56°C for 30 minutes before adding into bacterial culture. The mixed bacterial culture media of *P. acnes* and *S. aureus* were preincubated in the presence of 5% (v/v) anti-GFP (as a negative control) or anti-CAMP factor antiserum at 25°C for 1 hour before treatment. Each well of cells was cocultured with bacterial culture media in 5 µl at 37 °C for 24 hours. Cell viability was determined by an acid phosphatase assay as described previously (Nakatsuji *et al.*, 2009).

Statistical analysis

Data are presented as mean ± SE. Data are represented as means for measurement of lesion sizes. The Student *t*-test with criterion *P*<0.05 was used to determine statistical significance for all experiments. A two-way analysis of variance model was built to compare the treatment groups on the basis of log-transformed data with adjustment for the day of

experiments (two independent replicates) shown in Figure 5e. A Tukey's honest significant difference test (Miller, 1981) was performed for pairwise comparisons of the treatment groups at a family-wise error rate of 5%; the corresponding adjusted *P*-values were provided.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CAMP factor	Christie, Atkins, Munch-Peterson factor
CFU	colony-forming unit
GFP	green fluorescence protein
HA-MRSA	hospital-acquired MRSA
ICR	imprinting control region
MIP-2	macrophage inflammatory protein-2
MRSA	methicillin-resistant S. aureus
PBS	phosphate-buffered saline

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REFERENCES

Ahn CY, Ko CY, Wagar EA, et al. Microbial evaluation: 139 implants removed from symptomatic patients. Plast Reconstr Surg 1996;98:1225–1229. [PubMed: 8942908]

Bashir A, Mujahid TY, Jehan N. Antibiotic resistance profile: isolation and characterization of clinical isolates of staphylococci from patients with community-acquired skin infections. Pak J Pharm Sci 2007;20:299–304. [PubMed: 17604253]

Brook I, Frazier EH. Infections caused by *Propionibacterium species*. Rev Infect Dis 1991;13:819–822. [PubMed: 1962090]

Bubeck Wardenburg J, Schneewind O. Vaccine protection against *Staphylococcus aureus pneumonia*. J Exp Med 2008;205:287–294. [PubMed: 18268041]

Cogen AL, Nizet V, Gallo RL. Skin microbiota: a source of disease or defence? Br J Dermatol 2008;158:442–455. [PubMed: 18275522]

Cohen AL, Shuler C, McAllister S, et al. Methamphetamine use and methicillin-resistant *Staphylococcus aureus* skin infections. Emerg Infect Dis 2007;13:1707–1713. [PubMed: 18217555]

DeJonge M, Burchfield D, Bloom B, et al. Clinical trial of safety and efficacy of INH-A21 for the prevention of nosocomial staphylococcal bloodstream infection in premature infants. J Pediatr 2007;151:260–265. 265.e1. [PubMed: 17719934]

Diep BA, Gill SR, Chang RF, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet 2006;367:731–739. [PubMed: 16517273]

Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev 2000;13:16–34. [PubMed: 10627489]

Duncan SF, Sperling JW. Treatment of primary isolated shoulder sepsis in the adult patient. Clin Orthop Relat Res 2008;466:1392–1396. [PubMed: 18347887]

Gase K, Ferretti JJ, Primeaux C, et al. Identification, cloning, and expression of the CAMP factor gene (cfa) of group A streptococci. Infect Immun 1999;67:4725–4731. [PubMed: 10456923]

Goetghebeur M, Landry PA, Han D, et al. Methicillin-resistant *Staphylococcus aureus*: a public health issue with economic consequences. Can J Infect Dis Med Microbiol 2007;18:27–34. [PubMed: 18923684]

Grice EA, Kong HH, Conlan S, et al. Topographical and temporal diversity of the human skin microbiome. Science 2009;324:1190–1192. [PubMed: 19478181]

Hageman JC, Uyeki TM, Francis JS, et al. Severe community-acquired pneumonia due to *Staphylococcus aureus*, 2003–04 influenza season. Emerg Infect Dis 2006;12:894–899. [PubMed: 16707043]

Homa DG, Palfreyman MA. Infectious diseases in the operating room. CRNA 2000;11:8–14. [PubMed: 11271038]

Huseby M, Shi K, Brown CK, et al. Structure and biological activities of beta toxin from *Staphylococcus aureus*. J Bacteriol 2007;189:8719–8726. [PubMed: 17873030]

Jeong SH, Song YK, Cho JH. Risk assessment of ciprofloxacin, flavomycin, olaquindox and colistin sulfate based on microbiological impact on human gut biota. Regul Toxicol Pharmacol 2009;53:209–216. [PubMed: 19545513]

Jiang M, Babiuk LA, Potter AA. Cloning, sequencing and expression of the CAMP factor gene of *Streptococcus uberis*. Microb Pathog 1996;20:297–307. [PubMed: 9132527]

Jurgens D, Sterzik B, Fehrenbach FJ. Unspecific binding of group B streptococcal cocytolysin (CAMP factor) to immunoglobulins and its possible role in pathogenicity. J Exp Med 1987;165:720–732. [PubMed: 3546580]

Klevens RM, Gorwitz RJ, Collins AS. Methicillin-resistant *Staphylococcus aureus*: a primer for dentists. J Am Dent Assoc 2008;139:1328–1337. [PubMed: 18832268]

Kobayashi M, Tsuda Y, Yoshida T, et al. Bacterial sepsis and chemokines. Curr Drug Targets 2006;7:119–134. [PubMed: 16454704]

Lang S, Palmer M. Characterization of *Streptococcus agalactiae* CAMP factor as a pore-forming toxin. J Biol Chem 2003;278:38167–38173. [PubMed: 12835325]

Larsen HD, Aarestrup FM, Jensen NE. Geographical variation in the presence of genes encoding superantigenic exotoxins and beta-hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. Vet Microbiol 2002;85:61–67. [PubMed: 11792493]

Leyden JJ, McGinley KJ, Vowels B. *Propionibacterium* acnes colonization in acne and nonacne. Dermatology 1998;196:55–58. [PubMed: 9557227]

Liu YT, Lin SB, Huang CP, et al. A novel immunogenic spore coat-associated protein in *Bacillus anthracis*: characterization via proteomics approaches and a vector-based vaccine system. Protein Expr Purif 2008;57:72–80. [PubMed: 18029197]

Martin A, Clynes M. Comparison of 5 microplate colorimetric assays for *in vitro* cytotoxicity testing and cell proliferation assays. Cytotechnology 1993;11:49–58. [PubMed: 7763636]

McGinley KJ, Webster GF, Leyden JJ. Regional variations of cutaneous propionibacteria. Appl Environ Microbiol 1978;35:62–66. [PubMed: 623473]

McKenney D, Pouliot KL, Wang Y, et al. Broadly protective vaccine for *Staphylococcus aureus* based on an *in vivo*-expressed antigen. Science 1999;284:1523–1527. [PubMed: 10348739]

Miller LG, Perdreau-Remington F, Rieg G, et al. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N Engl J Med 2005;352:1445–1453. [PubMed: 15814880]

Miller, RG. Simultaneous Statistical Inference. New York: Springer; 1981. p. 300

Miller SI, Hoffman LR, Sanowar S. Did bacterial sensing of host environments evolve from sensing within microbial communities? Cell Host Microbe 2007;1:85–87. [PubMed: 18005684]

Nakatsuji T, Kao MC, Fang JY, et al. Antimicrobial property of lauric acid against *Propionibacterium acnes*: its therapeutic potential for inflammatory acne vulgaris. J Invest Dermatol 2009;129:2480–2488. [PubMed: 19387482]

Nishiwaki T, Yoneyama H, Eishi Y, et al. Indigenous pulmonary *Propionibacterium acnes* primes the host in the development of sarcoid-like pulmonary granulomatosis in mice. Am J Pathol 2004;165:631–639. [PubMed: 15277236]

Otto M. *Staphylococcus epidermidis* – the "accidental" pathogen. Nat Rev Microbiol 2009;7:555–567. [PubMed: 19609257]

Payne DJ. Microbiology. Desperately seeking new antibiotics. Science 2008;321:1644–1645. [PubMed: 18801989]

Ramage G, Tunney MM, Patrick S, et al. Formation of *Propionibacterium acnes* biofilms on orthopaedic biomaterials and their susceptibility to antimicrobials. Biomaterials 2003;24:3221–3227. [PubMed: 12763449]

Schneewind O, Friedrich K, Lutticken R. Cloning and expression of the CAMP factor of group B streptococci in *Escherichia coli*. Infect Immun 1988;56:2174–2179. [PubMed: 3294187]

Seybold U, Kourbatova EV, Johnson JG, et al. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. Clin Infect Dis 2006;42:647–656. [PubMed: 16447110]

Shi Y, Elmets CA, Smith JW, et al. Quantitative proteomes and *in vivo* secretomes of progressive and regressive UV-induced fibrosarcoma tumor cells: mimicking tumor microenvironment using a dermis-based cell-trapped system linked to tissue chamber. Proteomics 2007;7:4589–4600. [PubMed: 18022937]

Shinefield HR, Black S. Prevention of *Staphylococcus aureus* infections: advances in vaccine development. Expert Rev Vaccines 2005;4:669–676. [PubMed: 16221068]

Sohail M. A simple and rapid method for preparing genomic DNA from gram-positive bacteria. Mol Biotechnol 1998;10:191–193. [PubMed: 9819818]

Stranger-Jones YK, Bae T, Schneewind O. Vaccine assembly from surface proteins of *Staphylococcus aureus*. Proc Natl Acad Sci USA 2006;103:16942–16947. [PubMed: 17075065]

Takano T, Saito K, Teng LJ, et al. Spread of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals in Taipei, Taiwan in 2005, and comparison of its drug resistance with previous hospital-acquired MRSA. Microbiol Immunol 2007;51:627–632. [PubMed: 17579274]

Takizawa Y, Taneike I, Nakagawa S, et al. A Panton-Valentine leucocidin (PVL)-positive community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) strain, another such strain carrying a multiple-drug resistance plasmid, and other more-typical PVL-negative MRSA strains found in Japan. J Clin Microbiol 2005;43:3356–3363. [PubMed: 16000460]

Tancrede C. Role of human microflora in health and disease. Eur J Clin Microbiol Infect Dis 1992;11:1012–1015. [PubMed: 1295753]

Valanne S, McDowell A, Ramage G, et al. CAMP factor homologues in *Propionibacterium acnes*: a new protein family differentially expressed by types I and II. Microbiology 2005;151:1369–1379. [PubMed: 15870447]

Williams RE, Doherty VR, Perkins W, et al. *Staphylococcus* aureus and intra-nasal mupirocin in patients receiving isotretinoin for acne. Br J Dermatol 1992;126:362–366. [PubMed: 1533312]

Wilson PC, Rinker B. The incidence of methicillin-resistant *staphylococcus aureus* in community-acquired hand infections. Ann Plast Surg 2009;62:513–516. [PubMed: 19387151]

Yamada T, Eishi Y, Ikeda S, et al. *In situ* localization of *Propionibacterium acnes* DNA in lymph nodes from sarcoidosis patients by signal amplification with catalysed reporter deposition. J Pathol 2002;198:541–547. [PubMed: 12434425]

Yang D, Pornpattananangkul D, Nakatsuji T, et al. The antimicrobial activity of liposomal lauric acids against *Propionibacterium acnes*. Biomaterials 2009;30:6035–6040. [PubMed: 19665786]

Young VB, Schmidt TM. Overview of the gastrointestinal microbiota. Adv Exp Med Biol 2008;635:29–40. [PubMed: 18841701]
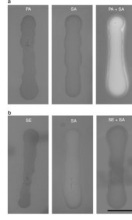
Figure 1. *Propionibacterium acnes*, not *Staphylococcus epidermidis*, enhances the hemolytic activity of *Staphylococcus aureus*
(**a**) *P. acnes* alone (PA, 2×10^7 colony-forming units (CFUs)), *S. aureus* 113 alone (SA, 2×10^7 CFUs), *P. acnes* plus *S. aureus* (PA + SA, 1:1 ratio with a total of 2×10^7 CFUs), (**b**) *S. epidermidis* alone (SE, 2×10^7 CFUs), or *S. epidermidis* plus *S. aureus* (SE + SA, 1:1 ratio with a total of 2×10^7 CFUs) were suspended in 10 µl PBS and streaked on sheep blood agar plates at 37°C for 2 days under anaerobic conditions. Hemolytic activity was determined as described in Materials and Methods. Bar = 1 cm. Data are representative of three separate experiments with similar results.
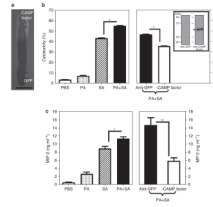
Figure 2. *Propionibacterium acnes* **Christie, Atkins, Munch-Peterson (CAMP) factor mediates the enhancement of hemolysis and cytolysis caused by a coculture of** *P. acnes* **with** *Staphylococcus aureus*

(**a**) A total of 10 µg of recombinant CAMP factor and green fluorescence protein (GFP) in 5 µl phosphate-buffered saline (PBS) was spotted (circles) adjacent to a *S. aureus* 113 (1×10^5 colony-forming units (CFUs) in 10 µl PBS) streak on a sheep blood agar plate grown overnight at 37°C. Bar = 1 cm. (**b**) Macrophage RAW264.7 cells were treated with PBS or bacterial culture supernatants (10 µg ml^{-1}) of *P. acnes* (PA), *S. aureus* (SA), or *P. acnes* plus *S. aureus* (PA + SA). Bacterial culture supernatants of coculture of *P. acnes* and *S. aureus* were incubated with 5% (v/v) anti-CAMP factor or anti-GFP (as a negative control) antiserum. The *in vitro* cytotoxicity assay was performed after cells were treated with the mixture of bacterial culture supernatant and antiserum for 24 hours. Western blot analysis was conducted to validate the specificity of anti-CAMP factor antiserum (arrowhead in the inset panel). One microgram of recombinant CAMP factor was separated via 12% SDS-PAGE, transferred to an Immobilon-P polyvinylidene difluoride membrane, and reacted with anti-CAMP factor (right lane) or anti-GFP antiserum (left lane), in 1:2,000 dilution. (**c**) Neutralization of CAMP factor significantly diminishes the enhancement of *P. acnes* on the *S. aureus*-induced macrophage inflammatory protein-2 (MIP-2) production. PBS (25 µl), *P. acnes* (PA, 25 µl; 2×10^7 CFUs), *S. aureus* 113 (SA, 25 µl; 2×10^7 CFUs), or a mixture of *P. acnes* plus *S. aureus* 113 (PA + SA, 25 µl; 1:1 ratio with a total of 2×10^7 CFUs) was intradermally injected into the ears of imprinting control region (ICR) mice. The mixture of *P. acnes* and *S. aureus* (PA + SA) was preincubated with 5% (v/v) anti-CAMP factor or anti-GFP antiserum at 25°C for 1 hour to neutralize the *P. acnes* CAMP factor. After incubation, the bacteria alone or bacteria preincubated with antiserum were intradermally injected into ears of ICR mice. After injection for 24 hours, ears were excised, homogenized, and centrifuged. ELISA was performed to measure the proinflammatory MIP-2 cytokine in supernatant. The data are represented as mean ± SE ($n = 3$, *$P<0.05$ and **$P<0.005$ by Student's *t*-test).
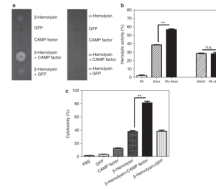
Figure 3. *Staphylococcus aureus* β-hemolysin contributes to the augmentation of hemolysis and cytolysis caused by coculture of *Propionibacterium acnes* and *S. aureus*

(**a**) Recombinant β-hemolysin (5 µg; Toxin Technology, Sarasota, FL), Christie, Atkins, Munch-Peterson (CAMP) factor (5 µg), and α-hemolysin (1 µg) in 5 µl phosphate-buffered saline (PBS) were spotted on sheep blood agar plates to examine the hemolytic activity. Recombinant green fluorescence protein (GFP; 5 µg) in 5 µl PBS was used as a control. (**b**) *P. acnes* (PA, 2×10^7 colony-forming units (CFUs)) alone, wild-type *S. aureus* alone (SAwt, 2×10^7 CFUs), or *P. acnes* plus wild-type *S. aureus* (1:1 ratio with a total of 2×10^7 CFUs) was incubated with sheep blood cells at 37°C with end-over-end rotation for 2 days. Hemolytic activity was detected by measuring the absorbance of hemoglobin release at 540 nm. To examine the essentiality of *S. aureus* β-hemolysin in the enhancement of hemolysis by coculture of *P. acnes* and *S. aureus*, a β-hemolysin-deficient *S. aureus* (SAhlb⁻, 2×10^7 CFUs) was incubated with sheep blood cells (PA + SAhlb⁻ in 1:1 ratio with a total of 2×10^7 CFUs) in the absence and presence of *P. acnes*. . (**c**) To determine the synergistic effect of CAMP factor and β-hemolysin on cytotoxicity, macrophage RAW264.7 cells (1×10^5 per well) were incubated with 10 µl of 200 µg ml^{-1} recombinant proteins of GFP, CAMP factor, β-hemolysin, or the mixture of β-hemolysin plus CAMP factor or GFP at 37°C for 18 hours. An equal volume of PBS was used as a negative control. After incubation, the cytotoxicity of recombinant proteins to macrophages was determined by an *in vitro* cytotoxicity assay as described in Materials and Methods. Data are represented as mean ± SE ($n = 3$, **P<0.005 by Student's *t*-test). NS, not significant.
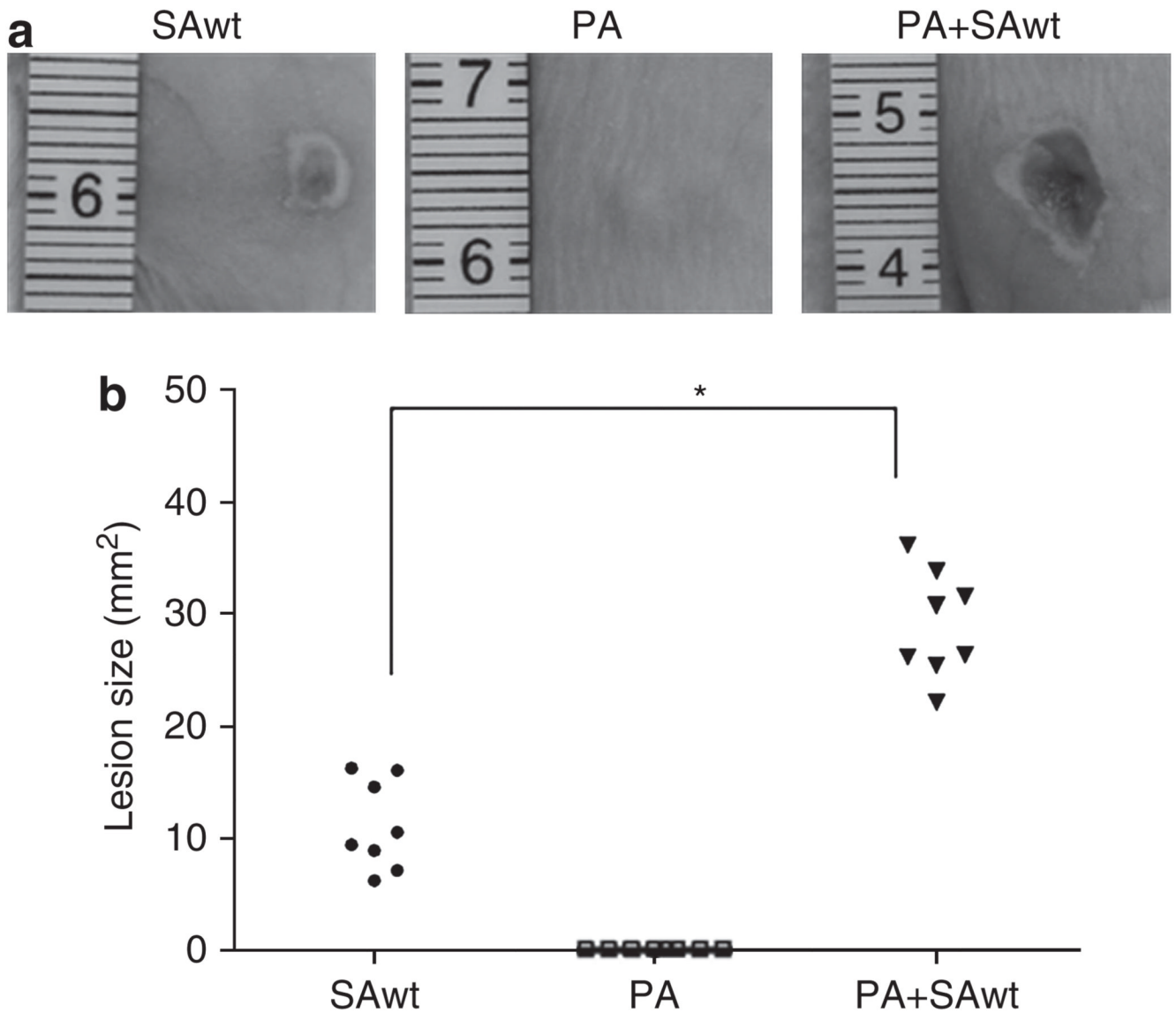
Figure 4. *Propionibacterium acnes* **intensifies** *Staphylococcus aureus*-**induced skin lesions**
(**a**) Wild-type *S. aureus* (SAwt, 1×10^7 colony-forming units (CFUs)), *P. acnes* (PA, 1×10^7 CFUs), or *P. acnes* plus wild-type *S. aureus* (PA + SAwt, 1:1 ratio with a total of 2×10^7 CFUs) in 50 µl phosphate-buffered saline was subcutaneously injected into the dorsal skins of imprinting control region mice to induce skin lesions. (**b**) Lesion sizes were examined and quantified 2 days after injection. Representative photographs of dorsal skin lesions are shown. Data are means of two independent experiments (*n* = 8, *P*<0.05 by Student's *t*-test).
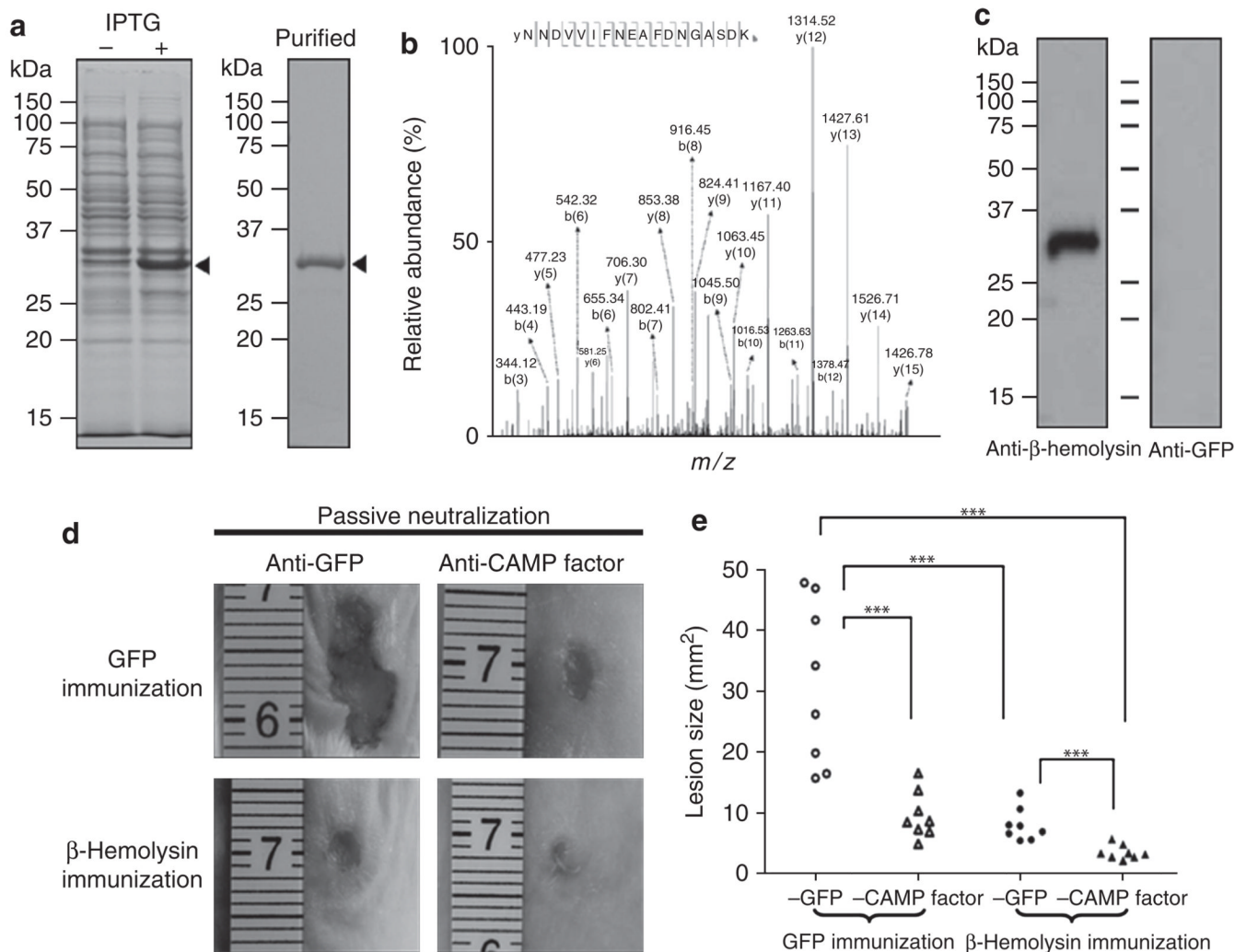
Figure 5. The immunogenicity of β-hemolysin and the combination of β-hemolysin vaccination and Christie, Atkins, Munch-Peterson (CAMP) factor neutralization confer immune protection against bacteria-induced skin lesions
(**a**) Recombinant β-hemolysin (arrowheads) was expressed in *E. coli* BL21 (DE3). Competent cells transformed with the pEcoli-Nterm 6 × HN vector-inserted cDNA encoding β-hemolysin were incubated without (−) or with (+) isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours and then subjected to 12% SDS-PAGE. Purified β-hemolysin is shown in the right panel. (**b**) The identity of recombinant β-hemolysin was analyzed by Nano liquid chromatography linear trap quadrupole tandem mass spectrometry (NanoLC-LTQ MS/MS). Tryptic digests of purified recombinant β-hemolysin were subjected to NanoLC-LTQ MS/MS. A sequenced internal peptide (NNDVVIFNEAFDNGASDK) of β-hemolysin is presented. The *m/z* value of each "y" and "b" ion in collision-induced dissociation spectra is indicated. All sequenced peptides are shown in Supplementary Table S1 online. (**c**) Immunogenicity of β-hemolysin was evaluated by western blotting. Imprinting control region mice were intranasally vaccinated with UV-inactivated *E. coli* BL21 (DE3) overexpressing β-hemolysin or green fluorescence protein (GFP). Anti-antisera were collected 1 week after second vaccination. One microgram of recombinant β-hemolysin was separated via 12% SDS-PAGE, transferred to an Immobilon-P polyvinylidene difluoride membrane, and reacted with anti-β-hemolysin (left lane) or anti-GFP antiserum (right lane),

in 1:2,000 dilution. (**d**) The GFP-(upper row) and β-hemolysin-immunized mice (lower row) were used for evaluation of immune protection. Mice were injected subcutaneously with the mixture of wild-type *Staphylococcus aureus* and *Propionibacterium acnes* (1:1 ratio with a total of 2×10^7 colony-forming units in 50 µl phosphate-buffered saline) that were preincubated with 5% (v/v) anti-CAMP factor antiserum (right panel) or anti-GFP antiserum (left panel) at 25°C for 1 hour. Lesion sizes were examined 2 days after injection. (**e**) Skin lesions were measured and statistics compiled. Representative photographs of dorsal skin lesions are shown. Data are from two independent replicated experiments (***P<0.001; Tukey's honest significant difference comparing the four treatments, in a two-way analysis of variance on log-transformed data comparing the four treatments, with day included as a blocking factor).