Vaccination Targeting a Surface Sialidase of *P. acnes*: Implication for New Treatment of Acne Vulgaris

Teruaki Nakatsuji\(^{1,2}\), Yu-Tsuen Liu\(^3\), Cheng-Po Huang\(^{2,3}\), Richard L. Gallo\(^{1,2}\), Chun-Ming Huang\(^{1,2,3,4} \)\*  

\(^{1}\) Division of Dermatology, Department of Medicine, University of California San Diego, San Diego, California, United States of America, \(^{2}\) Veterans Affairs (VA) San Diego Healthcare Center, San Diego, California, United States of America, \(^{3}\) Moores Cancer Center, University of California San Diego, San Diego, California, United States of America, \(^{4}\) La Jolla Institute for Molecular Medicine, San Diego, California, United States of America

Abstract

**Background:** Acne vulgaris affects more than fifty million people in the United State and the severity of this disorder is associated with the immune response to *Propionibacterium acnes* (*P. acnes*). Systemic therapies for acne target *P. acnes* using antibiotics, or target the follicle with retinoids such as isotretinoin. The latter systemic treatment is highly effective but also carries a risk of side effects including immune imbalance, hyperlipidemia, and teratogenicity. Despite substantial research into potential new therapies for this common disease, vaccines against acne vulgaris are not yet available.

**Methods and Findings:** Here we create an acne vaccine targeting a cell wall-anchored sialidase of *P. acnes*. The importance of sialidase to disease pathogenesis is shown by treatment of a human sebocyte cell line with recombinant sialidase that increased susceptibility to *P. acnes* cytotoxicity and adhesion. Mice immunized with sialidase elicited a detectable antibody; the anti-sialidase serum effectively neutralized the cytotoxicity of *P. acnes* in vitro and *P. acnes*-induced interleukin-8 (IL-8) production in human sebocytes. Furthermore, the sialidase-immunized mice provided protective immunity against *P. acnes* in vivo as this treatment blocked an increase in ear thickness and release of pro-inflammatory macrophage inflammatory protein (MIP-2) cytokine.

**Conclusions:** Results indicated that acne vaccines open novel therapeutic avenues for acne vulgaris and other *P. acnes*-associated diseases.

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*E-mail: chunming@ucsd.edu

Introduction

*P. acnes*, a gram-positive bacterium, is strongly associated with acne vulgaris. Isotretinoin, 13-cis-retinoic acid, is a vitamin A-derived retinoid that has been widely prescribed for systemic treatment of severe acne. However, the teratogenicity of isotretinoin is well documented [1,2]. Although isotretinoin was first approved in the United State in 1982 for treating severe acne, its use has become tightly regulated and it is not appropriate for most acne patients. Other therapies, such as systemic antibiotic treatments, also have limitations. These therapies may kill skin bacteria nonspecifically, impacting the homeostasis of resident dermal microflora [3,4]. In addition, oral antibiotics contain the risk of harming the intestinal microflora. The presence of *P. acnes* prevents colonization by more harmful bacteria (*Staphylococcus aureus* (*S. aureus*) and *Acinetobacter baumannii*), and it has been reported that *P. acnes* can transfer anti-bacterial resistance to other bacteria within the resident skin microflora when systemic antibiotic therapy is used [3]. Recently, antibiotic-resistant *P. acnes* has been found with the use of antibiotics [5]. Currently available topical treatments for acne lesions, including drugs, are palliative, requiring a sustained treatment regiment. When these therapies are discontinued, acne inevitably recurs. Acne vulgaris is a multi-factorial disease associated with *P. acnes* infection, hormone regulation and immune responses [1,3]. The inflammatory stage of acne vulgaris is usually the greatest concern to patients, as the lesions produced may lead to scarring and adverse psychological effects. Therefore, vaccines that suppress *P. acnes*-induced inflammation and pathogenesis, while minimizing the risk of altering the homeostasis of hormones and microflora, could be clinically valuable.

The complete genome of *P. acnes* has been sequenced [6]. Genomic data has revealed many gene encoded virulence factors, including sialidase, that are involved in degrading host tissue and inducing inflammation [7]. These virulence factors, which are either secreted from *P. acnes* or anchored in its cell wall, stimulate adjacent seocytes and keratinocytes, triggering acne inflammation. Sialidases of *P. acnes* can cleave sialoglycoconjugates to obtain sialic acids for use as carbon and energy sources [6]. Sialidase has been used previously as a vaccine target for several diseases, including influenza and bacterial pneumonia [8,9]. Thus, we chose a *P. acnes* surface sialidase (accession number: gi|50843035) containing an LPXTG cell wall-anchoring motif in the C-terminal domain [6,7] as a target for acne vaccine development. Our data demonstrated that sialidase-immunized mice demonstrated decreased *P. acnes*-induced ear swelling and reduced production of the pro-inflammatory cytokine MIP-2, providing a rational design
of acne vaccines that may offer a new treatment for acne vulgaris and other P. acnes-associated diseases.

Methods
Culture of P. acnes

P. acnes (ATCC® 6919) was cultured on Brucella broth agar, supplemented with 5% (v/v) defibrinated sheep blood, vitamin K, and hemin, under anaerobic conditions using Gas-Pak (BD Biosciences, San Jose, CA) at 37°C. A single colony was inoculated in Reinforced Clostridium Medium (Oxford, Hampshire, England) and cultured at 37°C until logarithmic growth phase. Bacterial pellets were harvested by centrifugation at 5,000 g for 10 min.

Molecular Cloning and Expression of Recombinant Sialidase

A polymerase chain reaction (PCR) product encoding a mature P. acnes cell wall anchored sialidase (accession number: gi|50843035) was generated using gene-specific primers designed based on the P. acnes complete genome sequence. The forward PCR primer (‘5′-TAAGGCCCTCTGGCAGCAACAGG-GTCCCCGGCCCGATGC-3′) included 16 nucleotides containing a Sal I site (GTGCAG) to match the end of the In-Fusion Ready pEcoli-Nterm 6xHN vector (Clontech Laboratories, Inc., Mountain View, CA), and 26 nucleotides encoding the N-terminal of the sialidase. The reverse PCR primer (‘5′-CAGAATTCGGCAAGCTTGTCTCCTGTGCGGCAAACTAG-3′) consisted of 16 nucleotides containing a Hind III site (AAGCTT) to match the end of the vector and 23 nucleotides encoding the C-terminal of the protein. PCR was performed using the forward and reverse primers and P. acnes genomic DNA as template. The amplified fragment was inserted into an In-Fusion Ready pEcoli-Nterm expression plasmid. Competent cells (E. coli, BL21 (DE3), Invitrogen, Carlsbad, CA) were transformed with this plasmid, selected on Luria-Bertani (LB)-plates containing ampicillin (50 µg/ml) and an isolated colony was cultured overnight at 37°C with gentle shaking. An aliquot of the overnight culture was diluted 1:20 with LB-medium and incubated at 37°C until reaching OD600 = 0.7. Isopropl-B-D-thiogalactoside (IPTG) (1 mM) was added into culture for 4 h to induce protein synthesis. Bacteria were harvested by centrifugation, rinsed with phosphate buffered saline (PBS), and suspended in 1/10 volume PBS. The bacteria were disrupted by sonication on ice for 1 min and lysed by centrifuging at 3,000 g for 30 min. The pellet was washed with PBS and dissolved in 50 mM sodium phosphate buffer containing 6 M guanidine HCl and 300 mM NaCl. The expressed protein possessing 6x HN tag was purified in denaturing condition with a TALON Express Purification Kit (Clontech Laboratories, Inc., Mountain View, CA). Subsequently, the purified protein was dialyzed against H2O, and then lyophilized. The lyophilized protein was dissolved in ethylene glycol (1 mg/1.2 ml), and then stirred in a refolding buffer (10 ml, 250 mM Tris-HCl buffer, pH 8.4, containing 5 mM cysteine, 0.5 mM cystine, and 1.5 M urea) at 4°C overnight. The refolded protein was dialyzed against PBS (pH 6.0), and concentrated. 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent gel staining with Coomassie blue were used for detection of protein expression.

Protein Identification via NanoLC-LTQ MS/MS Analysis

In-gel digestion with trypsin and protein identification via NanoLC-LTQ mass spectrometry (MS) analysis were performed essentially as described previously [10]. The automated NanoLC-LTQ MS/MS setup consisted of an Eksigent Nano 2D LC system, a switch valve, a C18 trap column (Agilent, Santa Clara, CA), and a capillary reversed phased column (10 cm in length, 75 µm i.d.) packed with 5 µm, C18 AQUASHIP resin with an integral spray tip (Picospritz, 15 µm tip, New Objective, Woburn, MA). A reversed-phase LC directly coupled to a LTQ ion trap mass spectrometer (Thermo Electron, Waltham, MA) was run using a linear gradient elution from buffer A (H2O plus 0.1% formic acid) to 50% buffer A plus 50% buffer B (acetonitrile plus 0.1% formic acid) in 100 min. The instruments were operated in the data dependent mode. Data on the four strongest ions above an intensity of 2×10^5 were collected with dynamic exclusion enabled and the collision energy set at 35%. Large-scale MS/MS spectra were extracted using default value by Bioworks® 3.2 (Thermo Scientific, San Jose, CA). Charge state deconvolution and deisotoping were not performed. All MS/MS spectra were analyzed using in-house Sorcerer™ 2 system with SEQUEST (v.27, rev. 11) as the search program for protein identification. SEQUEST was set up to search the target-decoy ipi.MOUSE.v3.14 database containing protein sequences in both forward and reverse orientations (68627 entries in each orientation) using trypsin as the digestion enzyme with the allowance of up to five missed cleavages. The false positive rates were roughly determined by doubling the ratio between the number of decoy hits and the total number of hits. SEQUEST was searched with a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 1.0 Da.

Sebocyte Culture and Cell Death Determination

An immortalized human sebocyte line S295 was obtained as a gift from Dr. Zouboulis CC in the Departments of Dermatology and Immunology, Dessau Medical Center, Dessau, Germany. Sebocytes were cultured in Sebomed Basal medium (Biochrom, Berlin, Germany), supplemented with 5 ng/ml human recombinant epidermal growth factor (Sigma, St. Louis, MO), 10% (v/v) heat-inactivated fetal bovine serum, at 37°C under atmosphere of 5% (v/v) CO2 in air. For determination of sialidase activity and the effect of sialidase on the sebocytes’ susceptibility to P. acnes infection, sebocytes (1.5 x 10^5) were incubated in a 96-well micro plate with 10 μg/ml of recombinant sialidase or green fluorescent protein (GFP) in the medium with the pH adjusted to 6.0 for 2 h. Incubation with the same volume of PBS served as a control. The sebocytes treated with sialidase or GFP were then co-cultured with P. acnes [multiplicity of infection (MOI) 1:10/cell: bacteria] for 18 h. After the co-culture, unbound bacteria were extensively washed three times with PBS. Dead sebocytes stained with trypan blue were counted on a hemocytometer. The colony forming unit (CFU) of P. acnes incorporated with sebocytes was determined by spreading serial dilutions of aliquots of trypsinized sebocyte suspension in 0.01% (w/v) Triton-X on agar plates to quantify CFU/cell. The adherence of P. acnes to sebocytes was visualized by staining with Accustain Gram stain kit (Sigma, St. Louis, MO).

Flow Cytometry

Sebocytes (1.5 x 10^6) were incubated with recombinant sialidase (10 μg/ml) at pH 6.0 for 2 h. The sebocytes were washed with PBS three times and fixed with 1% formaldehyde in PBS for 5 min at room temperature. After washing, the cells were incubated at 37°C for 15 min with 10 μg/ml of biotinylated MAA lectin I (Vector Laboratories, Burlingame, CA), which was prepared with 1% (w/v) bovine serum albumin (BSA) in PBS. The bound biotin was reacted with 1.5 μg/ml of a streptavidin-streptavidin-fluorescein isothiocyanate (FITC) conjugate (Jackson immunoresearch, West Grove, PA), which was incubated in 1% (w/v) BSA in PBS at 37°C for 10 min. After trypsinizing, the fluorescence intensities of the cells were analyzed with a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA).
Vaccination and Antibody Detection

Female ICR mice approximately 3-months-old (Harlan, Indianapolis, IN) were used for vaccination. Recombinant sialidase or GFP was dissolved in PBS and mixed with an equal volume of complete or incomplete Freund’s adjuvant. For the first vaccination, 50 μg of recombinant protein in complete Freund’s adjuvant was injected subcutaneously into the dorsal skin. Two weeks later, 50 μg of recombinant protein in incomplete Freund’s adjuvant was intraperitoneally injected for second boost. One week after the second boost, serum containing immunoglobulin G (IgG) antibody was harvested for western blot analysis. Serum was diluted 1:10,000 for the reaction. To obtain antisera against P. acnes, ICR mice were vaccinated intranasally with killed P. acnes (25 μl; 10⁷ CFU) for nine weeks (three boosts at three-week intervals). P. acnes was killed by heat at 65°C for 30 min or ultraviolet (UV) at 7,000 mJ/cm². Anti-serum raised against killed P. acnes was collected one week after the third boost. Each group (n = 4) and each experiment was performed in triplicate. 10% SDS-PAGE was used for western blot analysis. All experiments using mice were conducted according to institutional guidelines.

Immune Protection by A Sialidase-based Vaccine

Live P. acnes (20 μl; 10⁷ CFU) was subcutaneously injected in the central portion of the left ear of sialidase- or GFP-vaccinated mice. 20 μl of PBS was injected into the right ear as a control. After injection, ear thickness was measured using a micro caliper (Mitutoyo, Aurora, IL) and recorded periodically until ear swelling had nearly subsided (71 days). The P. acnes-induced change in ear thickness was calculated as % of that in PBS-injected ears. To assess the effect of vaccination on P. acnes growth, ears injected with PBS or live P. acnes in sialidase- or GFP-immunized mice were excised and homogenized at eight days after injection. P. acnes from homogenized ears were grown on agar plates for CFU counting.

Tissue Chamber and Pro-inflammatory Cytokine Detection

ICR mice were anesthetized with 10 mg of ketamine and 1.5 mg of xylazine per 100 g of body weight. The tissue chamber (internal and external diameters, 1.5 and 3.0 mm, respectively) consisted of closed polytetrafluoroethylene Teflon cylinders with 12 regularly spaced 0.1 mm holes. The tissue chamber was sterilized by soaking in 70% ethanol overnight. The sterile tissue chamber was then implanted subcutaneously under abdominal skin and maintained in the mice for 7 days to ensure the chamber was fully integrated with the subcutaneous environment. For histological observation, an implanted tissue chamber was cross-sectioned, stained with haematoxylin and eosin (H&E) (Sigma diagnostics, St. Louis, MO), and viewed on a Zeiss Axioskop2 plus microscope (Carl Zeiss, Jena, Germany). The fluorescence of MAA labeled-sialic acids in sialidase-treated sebocytes was reduced by approximately 69% (Figure 2A, a), whereas the fluorescence in control sebocytes treated with GFP was unchanged (Figure 2A, b). These data indicate that our purified recombinant sialidase enzymatically cleaves sialoglycoconjugates, releasing sialic acids. Treatment of purified sialidase for 2 h did not influence the cell viability of sebocytes (data not shown). After treatment with sialidase, or controls, for 2 h, sebocytes (5 × 10⁶ cells) were exposed to live P. acnes (5 × 10⁸ CFU) overnight. Live P. acnes induced approximately 15-20% cell death in PBS (vehicle)- or GFP-treated sebocytes, whereas sialidase-treated sebocyte cell death was significantly higher, at 33.5 ± 1.8 % (Figure 2B), suggesting that sialidase treatment increases the susceptibility of sebocytes to P. acnes.

The sebocytes were washed with PBS three times and reacted with β-NPP for 1 h at 37°C. The absorbance of the β-NPP reaction was measured at OD₄₀₀ nm. As a baseline, sebocytes killed with 0.1% (w/v) Triton-X were also assayed. Sebocyte death induced by P. acnes was determined with p-Nitrophenyl phosphate disodium (pNPP) (Pierce, Rockford, IL) according to the method of Martin and Clynes [11].
Sialidase was successfully expressed in E. coli. Sialidase (arrow) was expressed in E. coli in the absence (lane 1) or presence (lane 2) of (1 mM) IPTG. After IPTG induction, sialidase was successfully expressed in E. coli and shown at about 53.1 kDa on a 10 % SDS-PAGE (arrowhead). (B) Purified sialidase (arrow) was obtained via In-Fusion Ready TALON Express Bacterial Expression and Purification kit (Clontech Laboratories, Inc., Mountain View, CA). The expression and purity of sialidase were confirmed by Nano-LTQ MS/MS mass spectrometry (Thermo Electron Corp. Waltham, MA). A sequenced internal peptide (VVELSDTLMLNSR) of sialidase was presented. doi:10.1371/journal.pone.0001551.g001

Figure 1. Expression and Purification of A Cell Wall Anchored Sialidase. (A) A vector encoding a cell wall anchored sialidase (accession #gil50843035) was constructed by inserting a PCR amplified full sialidase gene into the pEcoli-Nterm 6xHN vector (Clontech Laboratories, Inc., Mountain View, CA) at the SaII and HindIII restriction sites. Specific primers including the sense (5'-ATGACTTTGACCACGAAACTGAGCG-3') and antisense primers (5’-TCAGCAGGCGCTCCGGCCCATGTC-3’) were designed to clone sialidase from P. acnes (ATCC 6919). The vector, which contains T7/LacO promoter, is derived from the pET system developed by William Studier and colleagues to achieve exceptionally high levels of protein expression in E. coli. Sialidase (arrow) was expressed in E. coli in the absence (lane 1) or presence (lane 2) of (1 mM) IPTG. After IPTG induction, sialidase was successfully expressed in E. coli and shown at about 53.1 kDa on a 10 % SDS-PAGE (arrowhead). (B) Purified sialidase (arrow) was obtained via In-Fusion Ready TALON Express Bacterial Expression and Purification kit (Clontech Laboratories, Inc., Mountain View, CA). The expression and purity of sialidase were confirmed by Nano-LTQ MS/MS mass spectrometry (Thermo Electron Corp. Waltham, MA). A sequenced internal peptide (VVELSDTLMLNSR) of sialidase was presented. doi:10.1371/journal.pone.0001551.g001

Immunogenicity in Mice Vaccinated with Recombinant Sialidase

To assess the immunogenicity of sialidase, ICR mice were vaccinated with heat killed P. acnes for nine weeks. Recombinant sialidase, GFP and sialidase lysates were subjected to western blot analysis. Many proteins with molecular weights greater than 50 kDa were immunoreactive with mouse serum obtained from the heat killed P. acnes-immunized mice (Figure 3A, lane 3), however, sialidase was not (Figure 3A, lane 1), indicating that mice immunized with whole organism P. acnes do not produce antibodies to sialidase. Similarly, recombinant sialidase was not immunoreactive to mouse serum obtained from UV-killed P. acnes-immunized mice (Figure S2), indicating that the undetected immunogenicity was not due to denaturation of sialidase during the heat treatment. We next vaccinated ICR mice with recombinant sialidase, or a GFP control, using Freund/incomplete adjuvants. Antibody production in the serum of immunized mice was detected by western blot analysis four weeks after immunization (Figure 3B, lane 1). A strong band appearing at 53.1 kDa was visualized when purified sialidase was reacted with serum from sialidase-immunized mice, indicating that sialidase was immunogenic in mice vaccinated with recombinant sialidase. No immunoreactivity against sialidase was detectable in GFP-immunized mice (Figure 3B, lane 2).

Protective Immunity Against P. acnes Challenge in Sialidase-vaccinated Mice

To assess immune protection in vivo, ICR mice immunized with recombinant proteins (sialidase or GFP) along with Freund/in-complete adjuvants were challenged with live P. acnes (10⁵ CFU) three weeks after vaccination. One ear of each mouse was subcutaneously injected with 25 μl of P. acnes (10⁷ CFU) and the other ear was injected with 25 μl of PBS as a control. Injection of P. acnes induced ear swelling (Figure 4A) and redness (Figure 4B). Ear thickness was measured regularly for 71 days, revealing a biphasic ear-swelling pattern. Ear thickness in GFP-immunized mice rapidly increased more than two fold (215.8±7.7%) 32 h after P. acnes challenge, decreased, then rebounded four days after challenge. Ear swelling was significantly reduced in both phases (at 32 hours and 4 days post-challenge) by more than 50% when mice were immunized with sialidase (Figure 4A). Sialidase immunization also resulted in decreased erythema in ears challenged with P. acnes (Figure 4B). Ear swelling in GFP-immunized mice nearly subsided 71 days after P. acnes challenge, whereas sialidase-immunized mice were completely recovered 58 days after challenge. These results indicate that sialidase-immunized mice suppressed P. acnes-induced ear inflammation.

Detection of Pro-inflammatory Cytokines in Implanted Tissue Chambers

Induction of pro-inflammatory cytokines also plays a key role in the progression of acne vulgaris. To determine whether sialidase immunization alters the level of P. acnes-induced pro-inflammatory cytokines, we employed a tissue chamber model (Figure 5A) to collect pro-inflammatory cytokines in vivo. The tissue chamber model has been extensively characterized in mice [13,14] and accurately mimics bacterial infections in vivo. A tissue chamber was implanted subcutaneously in the abdomen of ICR mice 7 days...
before \(P.\) \(acnes\) (\(10^7\) CFU) inoculation. Three days after \(P.\) \(acnes\) inoculation, tissue chamber fluids containing pro-inflammatory cytokines were drawn by percutaneous aspiration and the levels of MIP-2 (Figure 5B) and \((tumor\,\,necrosis\,\,factor)\,\,TNF-\alpha\) were measured by ELISA. In GFP-immunized mice, a significant increase in the level of MIP-2 was observed 3 days after \(P.\) \(acnes\) inoculation, while sialidase-immunized mice demonstrated 61% less induction. The level of TNF-\(\alpha\) remained unchanged after \(P.\) \(acnes\) inoculation in both GFP- and sialidase-immunized mice (data not shown). These results suggest that a vaccine targeting sialidase effectively suppresses \(P.\) \(acnes\)-induced production of the pro-inflammatory cytokine MIP-2 in the mice.
with similar results. Sebocytes stimulated with P. acnes that was pre-incubated with anti-GFP or anti-sialidase serum released IL-8 at the amount of 2.49±0.10 or 1.76±0.08 ng/ml, respectively (Figure 6B), indicating that anti-sialidase serum effectively suppresses P. acnes-induced IL-8 production in human sebocytes. Sebocytes within sebaceous glands are major target cells of P. acnes in patients with acne vulgaris [17]. Accordingly, generation of antibody against sialidase in acne patients may counteract the cytotoxicity of P. acnes to sebocytes and alleviate acne development.

Discussions

It has been reported that treatment of human buccal epithelial cells with the sialidase considerably increased Pseudomonas aeruginosa adherence [18]. In addition, immunization with recombinant Streptococcus pneumoniae sialidase resulted in a significant reduction in pneumococcal colonization in the chinchilla model [19]. We demonstrated that the adherence of P. acnes to human sebocytes was augmented after removal of sialic acids from the cell surface. This result is in agreement with previous findings that sialidase is involved in the adhesion of pathogens to host cells [18,20], and that treatment of host cells with sialidase changes their susceptibility to pathogens [19]. Adhesion process of bacteria occurs at the early stage of infection and is essential for its colonization, and in turn, colonization may be required for subsequent development of symptoms of diseases. Thus, vaccination targeting sialidase of P. acnes may be an efficient modality for the prevention of early infection of P. acnes.

Patients with acne lesions are likely to produce anti-P. acnes antibodies [21], however, acne lesions still recur in these patients. This suggests that patients infected with P. acnes may develop insufficient immunity to prevent subsequent P. acnes infection and acne recurrence. In this study, mice were immunized with either heat or UV killed P. acnes or recombinant sialidase. Our data has shown that sialidase is not immunogenic if vaccination with P. acnes is administered whereas sialidase becomes immunogenic when vaccination with recombinant protein is performed (Figure 3). An analysis of patients’ sera by western blot assay recognized a 96 kDa antigenic component of P. acnes [22]. No reports demonstrated that sialidase is antigenic in the sera of acne patients [21,22]. This result suggests that acne progression and recurrence could be effectively prevented if the antibody against sialidase of P. acnes can be robustly elicited in acne patients.

Inactivation of P. acnes has been used to create vaccines against acne vulgaris [23,24]. Acnevac or autovaccines containing killed strains of P. acnes and/or Staphylococci have been tested in acne and normal healthy subjects [25]. Although these killed P. acnes-based vaccines showed a good effect on acne patients, their effect is based on the non-specific modulation of the immune system of patients. Furthermore, It has been shown that mice immunized with killed P. acnes demonstrate non-specific resistance to challenge with other microbes [26]. A single intraperitoneal injection of phenol-treated P. acnes into mice showed non-specific resistance against subsequent lethal doses of an intraperitoneal challenge of Klebsiella pneumoniae, S. aureus, and Streptococcus pyogenes (S. pyogenes) [26]. Recently, it has been shown that animals sensitized with P. acnes exhibit an increased susceptibility to E. coli lipopolysaccharide (LPS) induced sepsis and [27] and liver failure [28]. The cell wall anchored sialidase presented in this article shares low identity with other surface sialidases in other pathogens [6]. Thus, acne vaccines
Figure 4. Immune Protection Conferred by A Sialidase-based Acne Vaccine. (A) The cytotoxicity of \( P. \) acnes was calculated as described in Methods and presented as mean±SE \( (P<0.0005^{**} \) by Student’s t-test). For assaying in vivo immune protection, ICR mice were immunized with recombinant sialidase or GFP using Freund (In)complete adjuvants (Figure 3). After confirming antibody production by western blot, live \( P. \) acnes \((10^7\) CFU, \(25\) μl) were injected subcutaneously into the ears of sialidase- and GFP-immunized mice, with PBS (25 μl) as a control. Ear thickness was periodically measured for 71 days after injection and changes reported as % of ear thickness in PBS-injected ears. \( P. \) acnes-induced ear swelling was significantly suppressed in sialidase-immunized mice in comparison with GFP-immunized mice \( (P<0.05^{*}) \), except for day 0, 4, 5 and 71. (B) Erythema was assessed in sialidase- (a) or GFP- (b) immunized mice 24 h after live \( P. \) acnes (left ears) or PBS (right ears) injection.

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Figure 5. A Sialidase-based Acne Vaccine Suppresses Induction of the Pro-inflammatory Cytokine MIP-2 by \( P. \) acnes. (A) A tissue chamber (a) (internal and external diameters, 1.5 and 3 mm, respectively, length, 1 cm; internal volume, 80 μl) was implanted subcutaneously in the abdomen of sialidase- or GFP-immunized mice. The tissue chamber consisted of closed polytetrafluoroethylene Teflon cylinders with 12 regularly spaced 0.1 mm holes. Bar: 1 cm. H&E staining of the cross-section of an implanted tissue chamber showed that the chamber was completely encapsulated by fibrotic tissue 7-days after implantation (b). Bar: 1.0 mm. \( P. \) acnes \((10^7\) CFU, \(20\) μl) was injected and trapped in this encapsulated tissue chamber. (B) Tissue chamber fluid containing MIP-2 was drawn by pecutaneous aspiration before (open bar) and three days after (solid bar) \( P. \) acnes injection. Measurement of MIP-2 was carried out by sandwich ELISA that used the Quantikine M mouse MIP-2 set (R&D System, Minneapolis, MN). Vaccination with sialidase markedly suppressed the \( P. \) acnes-induced increase in MIP-2. Error bars represented mean±SE of five separate experiments \(*P<0.005 \) by Student’s t-test).

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utilizing a P. acnes specific sialidase instead of killed P. acnes as the immunogen may be more specific and reduce the chance of side effects. There are at least five sialidases [sialidase B (gi|50843035); cell wall anchored sialidase (gi|50843035); sialidase A precursor (gi|50842172); putative sialidase (gi|50843278) and sialidase-like protein (gi|50843043) in P. acnes genome. Although creation of P. acnes with sialidase mutation or over-expression may directly address the role of sialidase in the virulence of P. acnes, it may be a challenge to genetically alter all sialidases in individual P. acnes. However, developing a novel compound to block the enzyme activities of all sialidases in P. acnes may be of value [29].

Our data has demonstrated that a sialidase-based acne vaccine provided protective effects on P. acnes-induced ear inflammation (Figure 4). Ear thickness was measured regularly for 71 days, revealing a biphasic ear-swelling pattern. This is consistent with previous results demonstrating a biphasic change in the activity of the mouse reticuloendothelial system after intraperitoneal injection with phenol-treated P. acnes [26]. The biphasic pattern can be explained by two distinct stages: short-term/local (early phase) and long-term/systemic (late phase) immune stimulation. The fluctuation in the number of macrophages and other host cells reflected a biphasic pattern of P. acnes infection. The effect of sialidase immunization on P. acnes growth was also explored. Ears injected with PBS or live P. acnes (10^7 CFU) in sialidase- or GFP-immunized mice were excised for homogenization eight days after bacterial challenge (data not shown). P. acnes from the homogenized ears were extracted and quantified on agar plates. The number of P. acnes in sialidase-immunized mice was not significantly different with that in GFP-immunized mice, indicating that sialidase immunization did not change the growth of P. acnes. Considering the data, the sialidase-based acne vaccine presented in this article may decrease P. acnes-induced inflammation without affecting the balance of body microflora.

Most animals including mice do not produce sufficient triglycerides in sebaceous glands to harbor P. acnes a fact that has encumbered the development of anti-acne vaccines and drugs targeting P. acnes infection [30]. Although Rhino mice with utricles that create larger follicles have been employed to determine compound comedogenicity [31], the genetic mutant mice cannot elicit antibodies against thymus-dependent antigens [32]. Thus, the use of Rhino mice as animal models may not be appropriate for vaccine evaluation. Rabbit ears have been utilized to determine compound comedogenicity of acne lesions [31]. However, the rabbit ear model has a lack of bacterial colonization and inflammation [33]. In addition, the use of rabbits may be inconvenient for vast vaccinations. A murine acne model measuring P. acnes-induced ear swelling and production of pro-inflammatory cytokines in tissue chambers may provide an alternative animal model for evaluating the potency of acne vaccines. Our data indicates that tissue chamber fluid contains various immune cells, including macrophages (CD11b+), neutrophils (Gr-1+), natural killer cells (CD49b+) and T cells (CD3+) (data not shown), suggesting an influx of immune cells into an implanted tissue chamber. In addition, IgG against P. acnes was detectable in tissue chamber fluids (data not shown) when tissue chambers were implanted into heat killed P. acnes-immunized mice. This result indicated that antibodies are able to migrate into tissue chambers to interact with injected P. acnes. By growing cells in a dermis-based cell-trapped system (DBCTS) and inserting into a tissue chamber, we successfully created a tissue microenvironment in vitro [34]. Thus, a bioengineering approach using a tissue chamber integrated with DBCTS may be able to create a humanized tissue microenvironment in animals to mimic the physiological structure of human hair follicles. The approach may eventually confer an animal model for evaluating vaccines targeting hair follicles. We detected two important murine pro-inflammatory cytokines (MIP-2 and TNF-α) (Figure 5). It has been reported that recognition of P. acnes by TLR2 induces the activation of pro-inflammatory pathways [28]. In vivo priming of mice with P. acnes also results in elevated serum levels of TNF-α [28]. The lack of elevated TNF-α levels in tissue chamber fluid after P. acnes inoculation may reflect a difference in host response between the tissue microenvironment (tissue chamber) and the systemic environment (serum).

Overall, we present a novel vaccine targeting cell wall anchored sialidase of P. acnes. Antibodies against sialidase provoked in vaccinated mice effectively suppressed the P. acnes-induced inflammation (Figures 4 and 5) and neutralized the cytotoxicity of P. acnes to human sebocytes (Figure 6), implicating that the sialidase-based
vaccine may have the potential for treatment of acne vulgaris, a most common skin disease affecting 85–100% of people at some point in their lives. In addition, the sialidase-based acne vaccine may be an alternative of the killed P. acnes-based vaccine that performed non-specifically and evoked many undesirable effects. Future directions include (i) establishing therapeutic acne vaccines that may benefit patients with severe acne and (ii) comparing the potency and side effects of sialidase-based vaccines with current medicines.

**Supporting Information**

**Figure S1** Quantitative analysis of the sialidase transcript in *P. acnes*. The gene expression of sialidase was determined by real-time quantitative PCR using specific primers as described in Methods. Total RNA isolated from anaerobically cultured *P. acnes* served as a template. The gene of triacylglycerol lipase known as a pathogenic factor of *P. acnes* was used as a positive control. A pGEM-T Easy Vector (Promega, Madison, WI) inserted with PCR products was performed to estimate the number of expressed genes. The level of gene expression of sialidase and triacylglycerol lipase was normalized to that of 16SrRNA gene.

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**Text S1**

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