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Phenotype and antimicrobial activity of Th17 cells induced by *Propionibacterium acnes* strains associated with healthy and acne skin

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Abstract

Studies of human skin microbiome suggest that *Propionibacterium acnes* strains may contribute differently to skin health and disease. However, the immune phenotype and functions of Th17 cells induced by healthy (P_H) vs. acne (P_A) skin-associated *P. acnes* strains are currently unknown. We stimulated PBMCs from healthy donors and observed that P_A strains induce higher IL-17 levels than P_H strains. We next generated P_H and P_A strain-specific Th17 clones and show that *P. acnes* strains induce Th17 cells of varied phenotype and function that are stable in the presence of IL-2 and IL-23. Although P_H and P_A-specific clones expressed similar levels of LL-37 and DEFB4, only P_H-specific clones secreted molecules sufficient to kill *P. acnes*. Furthermore, electron microscopic studies revealed that supernatants derived from activated P_H and not P_A-specific clones exhibited robust bactericidal activity against *P. acnes*, and complete breaches in the bacterial cell envelope were observed. This antimicrobial activity was independent of IL-26, as both natural IL-26 released by Th17 clones and rhIL-26 lacked antimicrobial potency against *P. acnes*. Overall, our data suggest that *P. acnes* strains may differentially modulate the $CD4^+$ T cell responses, leading to the generation of Th17 cells that may contribute to either homeostasis or acne pathogenesis.

Keywords

P. acnes; Protective Th17 cells; Ribotypes; Pathogenic Th17 cells; Interleukin 17

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Introduction

Acne vulgaris, is the most common inflammatory skin disease of the pilosebaceous unit (PSU) in humans. This disease affects millions of people worldwide, predominantly the pre-, adolescent and post-adolescent populations. (James, 2005; Williams *et al.*, 2012). *Propionibacterium acnes* is generally believed to play a major role in the pathogenesis of acne, in part by stimulating an inflammatory response. Other factors involved include, increased production of sebum, keratinocyte hyperproliferation, and altered *P. acnes* bacterial colonization (Leyden *et al.*, 1998; Zouboulis *et al.*, 2005). While most humans harbor *P. acnes* on their skin, not everyone suffers from acne. *P. acnes* is the dominant species within the microfollicles and the PSU in both healthy individuals and acne patients (Degitz *et al.*, 2007; Fitz-Gibbon *et al.*, 2013). However, at the strain level, *P. acnes* distribution has been shown to be significantly different in healthy and acne patients, suggesting that different *P. acnes* strains may play different roles in acne vulgaris (Fitz-Gibbon *et al.*, 2013).

Like other bacterial species, *P. acnes* shows phenotypic and genotypic diversity. This diversity at the strain level and its association with both human health and disease is poorly understood (Fitz-Gibbon *et al.*, 2013). Recently, typing of *P. acnes* has revealed associations of particular strains with different diseases (McDowell *et al.*, 2013a; Tomida *et al.*, 2013), and that various *P. acnes* strains may be associated with acne (referred to as P_A), while others may be associated with healthy skin (referred to as P_H) (Fitz-Gibbon *et al.*, 2013; Lomholt and Kilian, 2010; McDowell *et al.*, 2011). Strains of *P. acnes* have also been shown to have differences in pathogenic potential and secretome profiles (Holland *et al.*, 2010; Nakatsuji *et al.*, 2008; Suh and Kwon, 2015), as they differ in their ability to induce human β-defensin 2, influence cell growth, differentiation, and viability of skin resident cells and activation of both innate and adaptive arms of the immune response (Akaza *et al.*, 2009; Beylot *et al.*, 2014; Bojar and Holland, 2004; Nagy *et al.*, 2006; Nagy *et al.*, 2005).

A role for the adaptive immune response has also been suggested based on the detection of $CD4^+$ T cells in the inflammatory infiltrate from early acne lesions (Norris and Cunliffe, 1988). We and others recently demonstrated that *P. acnes* is a potent inducer of IL-17 and IFN-γ from $CD4^+$ T cells, and that IL-17^+ cells were present in perifollicular infiltrates in biopsies of inflammatory acne lesions, suggesting that acne may be a Th17-mediated disease (Agak *et al.*, 2014; Kistowska *et al.*, 2015; Thiboutot *et al.*, 2014). Moreover, Th17 cells not only characteristically induce the recruitment of neutrophils, which contribute to antibacterial activity, but also cause tissue injury. Reactive oxygen species and lysosomal enzymes are also released by neutrophils, and levels may correlate with severity (Abdel Fattah *et al.*, 2008; Chiu *et al.*, 2003; Levell *et al.*, 1989). Therefore, understanding the mechanisms by which P_A and P_H associated strains modulate the adaptive responses is essential to understanding the phenotypic differences of the strains and their roles in acne vulgaris.

In this study, we tested the hypothesis that *P. acnes* strains P_A and P_H induce Th17 cells with varied phenotypes and functions. The *P. acnes* strains P_A and P_H used in this study are representative of the ribotypes found to be strongly associated with healthy and acne-

associated skin and are shown in supplementary table 1 (Fitz-Gibbon *et al.*, 2013). We generated P_A and P_H -specific Th17 clones and evaluated the cytokine profiles and functional activity of Th17-derived molecules against *P. acnes in vitro*.

Results

P. acnes strains associated with acne disease (P_A) induce higher IL-17 levels than strains associated with healthy skin (P_H)

Microcomedones from healthy vs. diseased skin have been shown to harbor *P. acnes* strains from distinct lineages and possess distinct nucleopeptide signatures of 16S ribosomal DNA (rDNA) sequences (Fitz-Gibbon *et al.*, 2013). Although some *P. acnes* strains are found on healthy skin ribotype 6 (RT6), others have been associated with acne disease (RT4, RT5 and RT8) (McDowell *et al.*, 2013b). We sought to determine the cytokine secretion levels when PBMCs from healthy human donors were stimulated with P_H vs. P_A associated strains. We observed that P_A strains (HL110PA1, HL043PA1 and, HL096 PA1) induced significantly higher levels of IL-17 ranging from 1500-1700pg/ml in comparison to P_H associated strains (HLA042PA3, HL110PA3, HL110PA4), which induced IL-17 at a range of 750-1000pg/ml (Fig. 1a) ($P<0.001$). This P_A vs. P_H pattern of IL-17 induction was similar in all the donors that we tested.

Cloning efficiency and specificity of *P. acnes*-responsive $CD4^+$ $IL\text{-}17^+$ T cell clones

The observation that P_A strains induced significantly higher levels of IL-17 in comparison to P_H associated strains suggested that *P. acnes* strains might have the ability to modulate the immune response at the T cell level. In order to explore this possibility, we generated P_A and P_H-specific clones by stimulating PBMCs of four healthy donors with P_A and P_H associated strains as previously described (Agak *et al.*, 2014). Both P_A and P_H strains induced IL-17 secretion from PBMCs ranging from 1.5% -2% (Fig. 1b). We next sterile sorted and cloned the $CD4^+$ $IL\text{-}17^+$ population. We achieved a cloning efficiency ranging from 26 to 34% (Supplementary Table 2). We next selected antigen-specific T cell clones using T cell proliferation assays (Fig. 2a-b). Upon stimulation with α-CD3/α-CD28, both P_A and P_H-specific clones produced IL-17 ranging from 29-42%, and expressed Th17 associated genes (Fig. 2c-e). P_A and P_H-specific clones expressed functional markers of Th17 cells. In addition, treatment with α-HLA-DR antibody, but not with α-MHC I antibody, led to a strong reduction of T cell proliferation (Fig. 2f-g), indicating that proliferation and cytokine secretion by P_A and P_H-specific clones is dependent on MHC II. Thus, our T-cell cloning strategy can successfully generate $CD4^+IL\text{-}17^+$T-cells with desired specificities.

IL-23 is a stabilizing factor for *in-vitro* developed Th17 lineages

IL-23 has been shown to be important in the maintenance of Th17 cells (Veldhoen *et al.*, 2006). To specifically address the role of IL-23 as a stabilizing factor for the *in vitro* developed P_H and P_A specific clones, we tested the ability of the clones to survive *in vitro* both in the presence and absence of IL-23. In the absence of IL-23, the proportion of cells secreting IL-17 was sharply reduced on day 12 from 84% to 72%, and by day 40 all the clones lost the ability to secrete IL-17 (Fig. 3). Furthermore, upon stimulation with α-CD3/α-CD28 or APCs incubated/pulsed with microbe, these “exTh17” cells mainly secreted

IL-10 and IL-26 (Supplementary Fig. 1a). We next tried to rescue the IL-17 phenotype in the day 28 and 40 “exTh17” clones expressing 19% and 0% IL-17, respectively, which had been cultured without IL-23. Surprisingly, the loss of the Th17 phenotype was irreversible, as “exTh17” clones could not regain IL-17 protein secretion, even though both IL-23 treated and untreated clones demonstrated *IL-23R* expression as measured by qPCR (Supplementary Fig 1b-c). However, we noted no loss of IL-17 secretion in P_H and P_A -specific clones that were maintained in the presence of IL-23 (Supplementary Fig. 2).

P_H and P_A strains induce Th17 clones of varied phenotype

We developed a cloning system that uses the whole P_H and P_A microbes and autologous monocytes as APCs. This approach takes advantage of the complexity of the *P. acnes* microbes, which provides, at the same time, a large number of antigens and a variety of stimuli to innate receptors to elicit polarizing cytokines. Therefore, to investigate whether P_A and P_H -specific clones express and secrete functional molecules associated with Th17 cells, clones underwent costimulation by α-CD3/α-CD28 antibodies. After 24-hour stimulation, we observed that P_A and P_H strains induced similar levels of antimicrobial encoding genes, cathelicidin and *DEFB4* expression (Fig. 4a and supplementary Fig. 2a). Interestingly, P_A-specific clones expressed both Th17 and Th1 associated transcriptional factors *RORC* and *tbet*, in contrast to P_H- specific clones that only expressed *IL-17* (Fig. 4b). In addition, ELISA cytokine profiles indicate that P_A -specific clones secreted IFN-γ, IL-17, IL-22, and IL-26 whereas the P_H- specific clones secreted IL-10, IL-17, IL-22, and IL-26, and maintained this capacity at later time points (Fig. 4c-f and supplementary Fig. 2b-e). Non-stimulated clones did not secrete IFN-γ, IL-10, or any Th17 associated cytokine (data not shown). These findings suggest that P_H and P_A strains can modulate the T cell responses *in vitro* leading to induction of an IL-17/IFN-γ (IFN-γ^+IL-17^+IL-22^+IL-26^+) and/or the IL-17/IL-10 (IL-10^+IL-17^+IL-22^+IL-26^+) producing phenotypes.

Not all Th17 clones secrete molecule(s) that are sufficient to kill *P. acnes*

Given the importance of IL-26 as a cationic amphipathic protein that is able to kill extracellular bacteria via membrane pore formation (Meller *et al.*, 2015), we next sought to determine whether Th17-derived molecules had microbicidal activity against *P. acnes*. We conducted CFU assays as previously described (McInturff *et al.*, 2005; Schmidt *et al.*, 2015) using supernatants derived from activated P_H and P_A-specific clones. All Th17 clones derived from P_H-associated strains exhibited robust bactericidal activity against *P. acnes* strains that we tested (Fig. 5a and supplementary Fig. 3a-b). Against *P. acnes* strains HL096PA1 (Fig. 5a), greater than 5-log reductions in CFU were observed at a dilution of 1:4. We used the antimicrobial peptide LL-37 as a positive control. LL-37 (0.1-100 μM) was also effective against all strains of *P. acnes*. (Fig. 5b). In contrast, no single clone derived from P_A-associated strains exhibited microbicidal activity against *P. acnes.* Even though we observed a 1-log reduction in CFU using neat supernatants for some P_A-specific clones, including P_A-1, this was not significant (Fig. 5c). We therefore classified the IL-10^+ IL-17^+ IL-22^+ IL-26^+-secreting clones as protective Th17 (prTh17) and the IFN-γ^+ IL-17^+ IL-22^+ IL-26^+-secreting clones as pathogenic Th17 (paTh17) as our data corroborate recent evidence that broadly categorize ROR$^+$Th17 cells into two groups: first, host protective cells Th17 that express both IL-17 and IL-10 (Esplugues *et al.*, 2011; Hirota *et al.*, 2013;

McGeachy *et al.*, 2007) and, second, a highly inflammatory population that express IL-17, IL-22 and IFN-γ (Langrish *et al.*, 2005; Zheng *et al.*, 2007).

We next used electron microscopy to determine whether supernatants from activated prTh17, like other antimicrobial peptides, kill bacteria by pore formation and membrane disruption. Transmission EM micrographs of untreated *P. acnes* illustrate their normal pleomorphic structure (Supplementary Fig. 4a). After a 3-hour treatment with supernatants derived from activated clone P_H-1 (prTh17), we observed complete breaches in the cell envelope, with leakage of cytosol to the extracellular environment (Fig. 5d). The same microbicidal activity was observed for LL-37 (Fig. 5e). We further observed that the killing molecule(s) in these supernatants was heat sensitive, as the antimicrobial activity was completely abrogated by heat inactivation (Supplementary Fig 3d), and that IL-26 neutralizing antibody had little to no effect on the ability of prTh17 clones to kill *P. acnes in vitro* (Supplementary Fig 3e-f). In contrast, P_A-1 (paTh17) derived supernatants had no effect on *P. acnes* (Fig. 5f). We further observed that both P_A and P_H strains induce IL-26 secretion in PBMCs (Supplementary Fig. 4b) and that IL-26 was highly expressed in both paTh17 and prTh17 clones (Supplementary Fig. 4c). We therefore assessed whether the phenotypic characteristics of paTh17 and prTh17 would be correlated with IL-26 function as IL-26 has recently been described as an antimicrobial protein that efficiently kills extracellular bacteria (Meller *et al.*, 2015). We validated that the antimicrobial activity against *P. acnes* by prTh17 cells was not due to the action of IL-26, as both natural IL-26 released by a Th26 clone and rhIL-26 lacked antimicrobial potency against *P. acnes* in CFU assays (Supplementary Fig. 4d-f). In further experiments, we examined whether this molecule(s) was effective against other Gram positive and negative bacteria. As previously reported (Meller *et al.*, 2015), we observed an inhibitory or killing effect when *E. coli* and *S. aureus* were incubated with both natural IL-26 released from a Th26 clone and increasing doses of rhIL-26 (Supplementary Fig. 5).

Discussion

The link of Th17 cells to pathologic inflammation is very well established in comparison to our understanding of the role of these cells in host defense. Several studies support their involvement in protection against extracellular bacterial infections such as *Klebsiella pneumoniae* in mice (Aujla *et al.*, 2008; Happel *et al.*, 2005). However, in humans indirect evidence has recently emerged demonstrating that patients suffering from diseases such as hyper-IgE syndrome, and Mendelian susceptibility to mycobacterial diseases, may have defects in the Th17 cells/pathway (Boniface *et al.*, 2008; Eyerich *et al.*, 2008; Milner *et al.*, 2008; Renner *et al.*, 2008). In addition, both IL-17 and IL-22 induce antimicrobial peptide production from epithelial cells in humans (Liang *et al.*, 2006; Ouyang *et al.*, 2008; Wilson *et al.*, 2007). More recently, Th17-derived IL-26 was shown to have direct antimicrobial activity suggesting their participation in host defense (Meller *et al.*, 2015). Here, we generated P_A- and P_H-specific clones against six strains of *P. acnes* and demonstrate that these strains have an ability to differentially modulate the $CD4^+$ T cell responses. Importantly, we show that IL-23 is a stabilizing factor for *in vitro* developed Th17 lineages, and that prTh17 but not paTh17 secrete molecules that are sufficient to kill *P. acnes in vitro*. Together, our data suggest that certain subsets of Th17 cells may be involved in providing protection against *P. acnes*.

Our observations that certain P_A strains induced higher IL-17 levels than P_H strains suggest that *P. acnes* strains express different antigenic components/ligands on their surface structure which may be responsible for the differential Th17 responses. These observations corroborate recent results of complete genome studies that compared the genomes of P_A associated strain HL096PA1 and that of P_H associated strain KPA171202. The studies revealed that, present in HL096PA1 and absent in KPA171202, was a plasmid carrying a tight adhesion locus, which has been shown to enhance colonization and biofilm formation (Kachlany *et al.*, 2000; Tomich *et al.*, 2007). In addition, HL096PA1 also contained genomic islands that were unique to P_A-associated strain and may be associated with increased virulence of this lineage in acne (Fitz-Gibbon *et al.*, 2013; Kasimatis *et al.*, 2013). The identification of surface antigens/ligands expressed in P_A vs. P_H strains that activate PRRs on APCs leading to secretion of inflammatory cytokines, may potentially lead to new diagnostic and therapeutic approaches against acne.

Notably, both paTh17 and prTh17 clones expressed similar levels of antimicrobial proteins cathelicidin and DEFB4. However, despite secreting high levels of IL-26, paTh17 clones demonstrated no antimicrobial activity against *P. acnes*. This result was surprising as *in vitro* derived IL-26 was recently shown to be a cationic and amphipathic cytokine that could effectively kill both Gram positive and Gram negative bacteria (Meller *et al.*, 2015). In contrast, prTh17 clones, secreted molecule(s) that were sufficient to kill *P. acnes*, and we envisage that the high potency of this molecule(s) may be due to synergy with other additional molecules in the culture supernatant.

Th17 cells play a crucial role in controlling different microorganisms *in vivo* (Khader *et al.*, 2009). We used *P. acnes* as a model to understand the nature of Th17 responses that may exist in acne vulgaris. Our findings highlight the fact that P_H and P_A strains differentially modulate the $CD4^+$ T cell responses *in vitro* leading to induction of IL-17/IFN-γ (paTh17) and IL-17/IL-10 (prTh17) producing cells, and suggest that divergent Th17 responses may exist in acne. Th17 cells have been intensively studied in immune-mediated diseases, but the physiological function of the IL-17/IFN-γ and/or IL-17/IL-10 axis in the context of acne is unknown. Heterogeneous highly inflammatory (Langrish *et al.*, 2005; Zheng *et al.*, 2007) and host protective Th17 responses (Esplugues *et al.*, 2011) have previously been described. In our study, the paTh17 phenotype was mainly induced by P_A strains and we associated this phenotype with acne pathogenesis, whereas the P_H strains induced the prTh17 phenotype which we associated with protection since activated prTh17 cells demonstrated microbicidal activity against *P. acnes in vitro*. These data are supported by a previous report that demonstrated that *in vitro* generated non-pathogenic Th17 cells are able to express IL-10 (McGeachy *et al.*, 2007). Of note, Th17 cells have been shown to respond not only to canonical Th17-dependent pathogens such as *Candida albicans*, but also to Th1-inducing, intracellular pathogens such as *Mycobacterium* (Acosta-Rodriguez *et al.*, 2007). Although further studies are required to delineate the mechanisms by which *P. acnes* strains drive the Th17 to Th1 differentiation program and vice versa, it is likely that poly-functional IL-17/IL-10, but not IL-17/IFN-γ secreting Th17 cells may be beneficial in containing *P. acnes*. A better mechanistic understanding of how *P. acnes* strains modulate immune responses can inform future microbiome studies and probiotic designs.

In summary, we demonstrate that *P. acnes* strains differentially modulate the $CD4^+$ T cell responses, leading to the generation of Th17 cells that may contribute either to homeostasis or pathogenesis in acne vulgaris. It is important to highlight that although RT6 may have only been found on healthy skin to date, the number of subjects have been few, and since current studies indicate that RTs 1, 2 and 3 seem to be evenly distributed in healthy and acne skin, additional studies comparing these *P. acnes* ribotypes directly isolated from either healthy or acne skin would give a better indication of how acne disease state can be linked to the different functional T cell responses. Our findings and further studies aiming to identify the underlying mechanism(s) by which distinct strains of *P. acnes* trigger divergent Th17 responses may lead to identification of alternative targets in therapy for acne.

Materials and Methods

Bacterial strains

P. acnes strains used in the study are highlighted in (Supplementary Table 1). P_A strains (HL110PA1, HL096PA1, HL043PA1), and P_H strains (HL042PA3, HL110PA4, HL110PA3) were obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and cultured as previously described (Agak *et al.*, 2014). The level of endotoxin contaminating the *P. acnes* was quantified with a *Limulus* Amoebocyte Lysate assay (BioWhittaker, Radnor, PA) and found to be <0.1 ng/ml^{-1}. *Staphylococcus aureus* SA113 and *Escherichia coli* DH5α were grown in Luria broth (LB) overnight at 37°C with agitation. Overnight bacterial cultures were subcultured and incubated until midlog was reached, which was determined to be OD_{600} = 0.4. Cultures were washed in sterile PBS and renormalized to OD_{600} = 0.4 in culture media.

PBMC isolation, stimulation and cytokine ELISAs

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors with written informed patient consent, as approved by the University of California, Los Angeles Institutional Review Board. PBMCs were then isolated using Ficoll–Paque gradients (GE Healthcare) as previously described (Agak *et al.*, 2014). Briefly, cells were cultured in T cell media (RPMI 1640, 10% heat inactivated human serum (Gemini), 2mM L-glutamine, 10U/ml penicillin and 100 μg/ml streptomycin) and stimulated with different strains of *P. acnes* at 1 multiplicity of infection (1 MOI). Levels of cytokines accumulated in culture supernatants were measured by ELISA. Samples were assayed in triplicates. Monocyte isolation from PBMC was done using the Monocyte Isolation Kit (Miltenyi Biotec, Auburn, CA) following manufacturer's protocol.

Reagents

IL-4 (BD555194), IL-10 (DY217b), IFN-γ (DY285), IL-13 (DY213), IL-17 (DY317), IL-22 (DY782), IL-26 (CSB-E11716h), rhIL-26 (R&D), rhIL-23 (R&D), rhIL-2 (R&D), Blocking mouse anti human HLA-Class I (W6/32) and anti-HLA-C (Abcam) both used at 10μg/ml, anti-MHC-II (MS163P1ABX) (Fisher), anti-IL-26 mAb1375, clone 197505 (R&D), CD4 (OKT4) (BD), IL-17PE (BD), IFN-γ- FITC (BD), CD14 FITC (BD) LL-37 (Innovagen). Mouse IgG1 and IgG2b isotype matched antibodies were used as controls.

Sterile Cell sorting

PBMCs from healthy donors were stimulated for 16 hours with P_H and P_A strains, and IL-17 secretion determined using a cytokine secretion capture assay (Cell Enrichment and Detection Kit) following the manufacturer's protocol (Miltenyi). After IL-17 staining, the cells were further stained with α-CD4 antibodies. $CD4^+$ $IL\text{-}17^+$ cells were sorted under sterile conditions using Beckton Dickinson FACS Vantage (San Jose, CA). Dead cells were excluded by DAPI staining. Data acquisition and analysis was done using FlowJo software (V7.6).

T cell cloning and proliferation assays

Sorted cells were cloned at 0.3cells/well in Terasaki plates (Nunc Microwell, Sigma-Aldrich) in the presence of unmatched-γ-irradiated (10^6) PBMCs activated by 2μg/ml phytohaemagglutinin (PHA-Murex) in T cell media supplemented with 100 U/ml rhIL-2. Plates were screened from days 8-12 post cloning and growing clones were transferred to 96 well flat-bottom plates and later expanded in 24 well plates in T cell media supplemented with 100 U/ml IL-2 and 2ng/ml IL-23. Specificity of the clones was confirmed using proliferation assays. Briefly, $CD4^+IL\text{-}17^+$ T cell clones at 5×10^4 cells/well were seeded into 96-well flat-bottom microplates (BD) containing 2.5×10^4 autologous monocytes in T cell media with/without *P. acnes* strains (1 MOI) that was originally used as a stimulus to generate the clones. ^{3}[H] thymidine (1 uCi/well) was added 4 h before the culture was terminated. The cells were harvested and assayed by scintillation counting.

T cell stimulation and Flow cytometry

For analysis of cytokine production, T cell clones were stimulated with either control medium or with α-CD3/α-CD28 (BD) in the presence of GolgiPlug (BD). Cells were next stained, and examined by flow cytometry. Samples were acquired on BD Biosciences FacsScan, and analyzed using FlowJo software (V7.6).

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated from T cell clones after polyclonal activation using Trizol reagent (Invitrogen) following manufacturer's protocol and treated with RNase-free DNase. cDNA and real-time PCR was done as previously described (Agak *et al.*, 2014). *GAPDH* was used as a control. Gene expression level was quantified by the comparative method $2^{-\Delta\Delta CT.}$ The list of primers used in the study are summarized in Supplementary table 3.

CFU assay

P. acnes strains (Supplementary Table 1) were grown under anaerobic conditions in Reinforced Clostridial Medium (Oxoid, Basingstroke, England) for 2 d and collected in mid-log phase. The bacteria were washed three times with the assay buffer (10 mM Tris pH 7.4, supplemented with 0.03% volume trypticase soy broth, Tris-TSB), and enumerated by applying a conversion factor of 7.5×10^7 bacteria per mL=1 OD_{600}. Th17 culture supernatants were diluted in Tris-TSB and the CFU assays performed as previously described (McInturff *et al.*, 2005; Schmidt *et al.*, 2015). For the *S. aureus* and *E. coli* CFU assays, bacteria were grown as described above and resuspended in RPMI 1640. Depletion

of IL-26 was performed by incubating supernatants with 10ug/ml of neutralizing anti-IL-26 mAb or an isotype mAb for 12 h at 4°C. 100μl reactions (bacteria + Th17 supernatants or rhIL-26 or anti-IL-26) were added to 1.5-ml tubes and incubated at 37°C with shaking for 1, 3, or 24 h after the specified incubation periods, 10-fold serial dilutions were plated on LB plates to quantify surviving CFU.

Electron Microscopy

P. acnes strains at 3×10^8 CFU/mL were incubated untreated or with supernatants (1:2 dilutions for prTh17 and 1:2 and neat for paTh17) derived from activated clones, LL-37, or rhIL-26 for 3 h, washed twice with PBS, and resuspended in PBS with 2% glutaraldehyde. Samples were processed as previously described (Schmidt *et al.*, 2015).

Statistical analysis

Results are expressed as the means ± SD for the number of separate experiments indicated in each case ($n\geq3$). One-way analysis of variance was used to compare variances within groups and among them. *Post hoc* two-tailed Student's *t*-test was used for comparison between two groups. Significant differences were considered for those probabilities ≤ 5% ($P\leq0.05$)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PBMCs	Peripheral blood mononuclear cells
prTh17	Protective Th17 cells
paTh17	Pathogenic Th17 cells
P_A	*P. acnes* strains associated with acne
P_H	*P. acnes* strains associated with healthy skin
CFU	Colony forming units

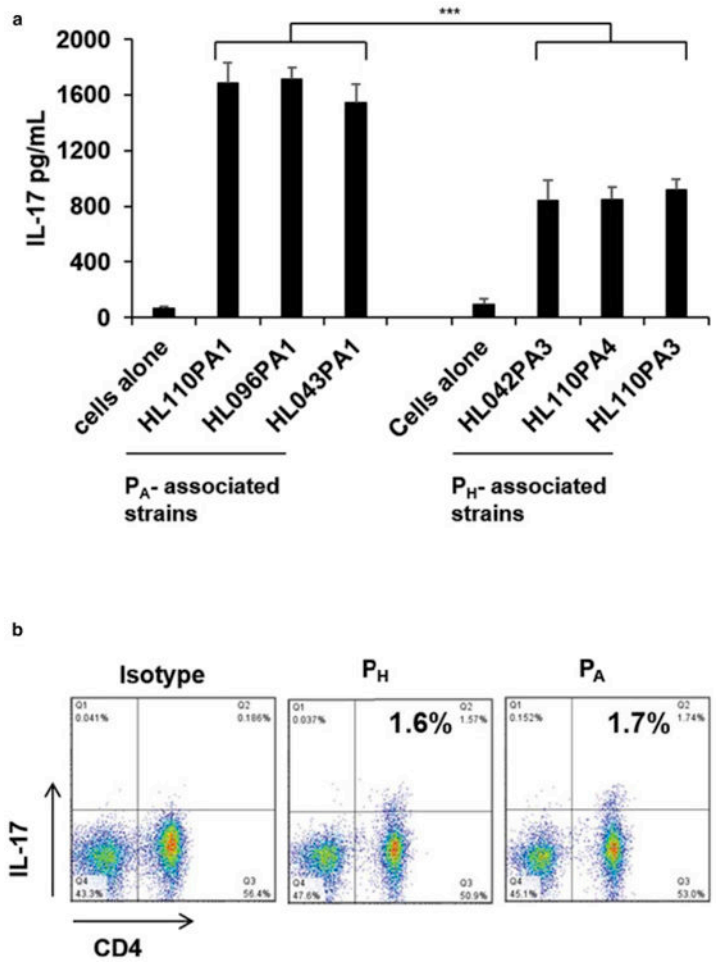

Fig. 1. *P. acnes* strains associated with acne disease induce significant IL-17 responses

(a) PBMCs were cultured (2-5 × 10^6/well) in the presence of live P_A and P_H strains (1MOI). Levels of IL-17 accumulated in the culture supernatants were measured using ELISA. Results from three donors were combined and the variation demonstrated as mean ± SD. (***$p \leq 0.001$ compared to P_H associated strains). **(b)** For Sterile cell sorting, PBMCs were stimulated with P_H and P_A strains (1MOI) for 16 h and IL-17 secretion determined using a cytokine secretion capture assay. The cells were further stained with α-CD4 antibodies, and the $CD4^+IL\text{-}17^+$ cells were sorted under sterile conditions and cloned at 0.3cells/well. Each panel is representative of four independent experiments.

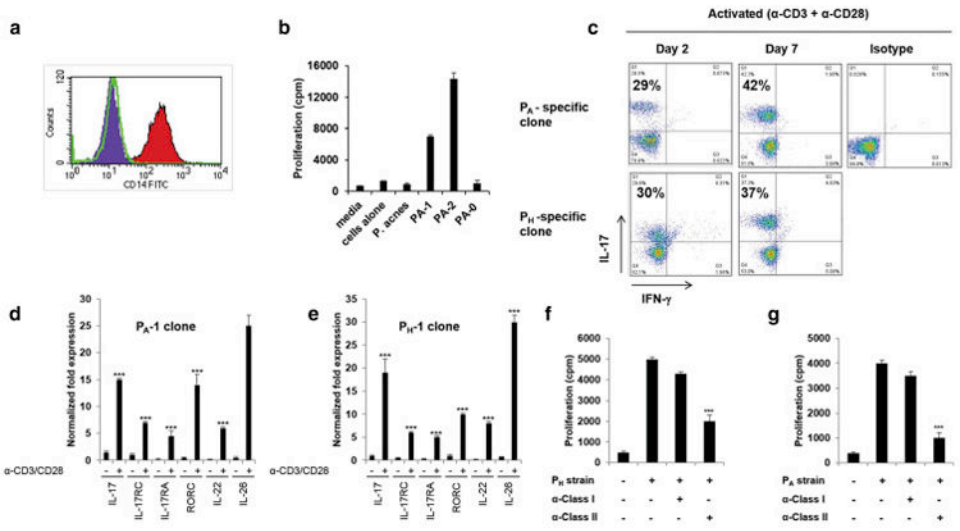

Fig. 2. P_H and P_A Th17 clones are specific and express Th17 associated genes

(a) Autologous $CD14^+$ monocytes were isolated by positive selection and used as APCs. **(b)** Specificity of P_A and P_H specific clones as determined by ^{3}[H] thymidine incorporation in T cell proliferation assays. **(c)** FACS on clones stimulated with a-CD3/a-CD28 (0.75μg/ml) and stained with antibodies to IL-17 and IFN-γ. **(d-e)** Real-time PCR of Th17 associated genes analyzed 6 h following (-) no or α-CD3/α-CD28 stimulation. Gene expression was normalized to the housekeeping gene *GAPDH* and quantified by the comparative method $2^{-\Delta\Delta CT}$, (n=4). (**f-g**) Effects of blocking MHC class I and II (10μg/ml) in autologous monocytes as measured in T cell proliferation assays. Data represent mean ± SD (***$p \leq 0.001$).

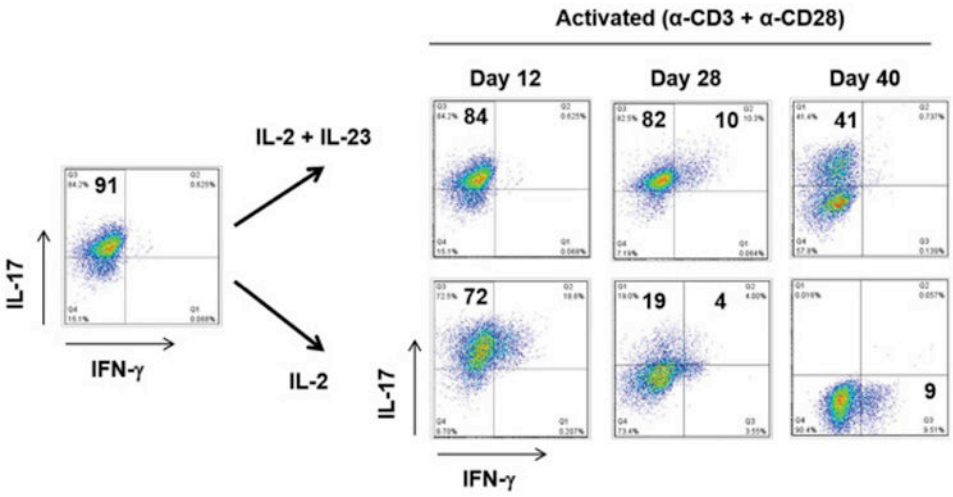

Fig. 3. IL-23 is a stabilizing factor for *in-vitro* developed P_A and P_H- specific Th17 clones

P_A and P_H- specific clones cultured with IL-2 and with/without IL-23 were stimulated with α-CD3/α-CD28 (0.75μg/ml) on days 12, 28, and 40 and stained with antibodies to IL-17 and IFN-γ. Data is representative of more than five independent experiments using clones derived from four different donors.

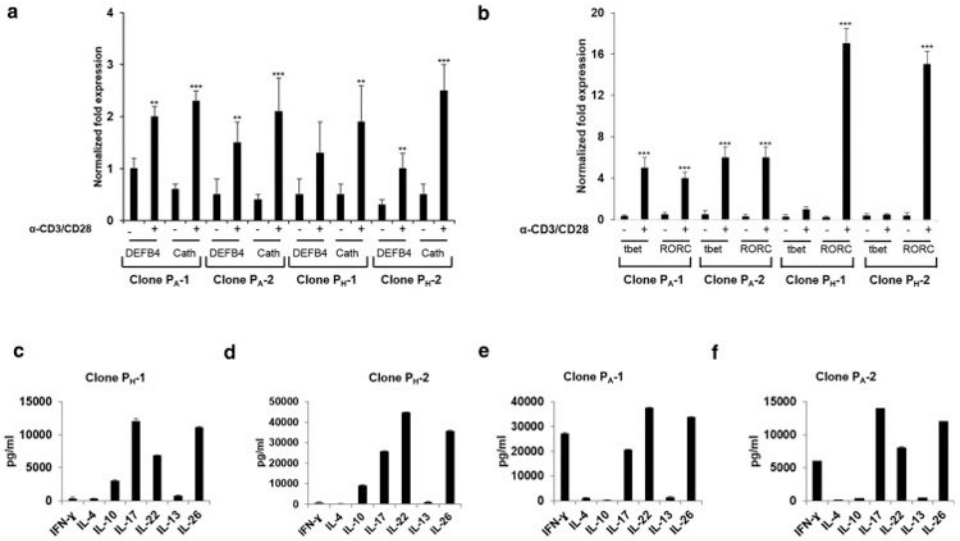

Fig. 4. Gene expression and cytokine profiles of P_A and P_H-strain specific clones

(**a-b**) *DEFB4, Cathelicidin (Cath), tbet,* and *RORC* analyzed 24 h following (-) no or α-CD3/α-CD28 (0.75μg/ml) stimulation representative prTh17 clones (P_H-1 and P_H-2) and paTh17 (P_A-1 and P_A-2) generated from two different donors (D1 and D2) are shown. The results were also consistent for prTh17 and paTh17 clones generated from donors D3 and D4 (Supp. Fig 2). Gene expression was normalized to GAPDH. Data are representative of three independent experiments (n=3). Data represent mean ± SD (**$p \leq 0.05$; ***$p \leq 0.001$). (**c-f**) P_A and P_H-strain specific clones were stimulated as above, and cytokine levels accumulated in the culture supernatants were measured using ELISA.

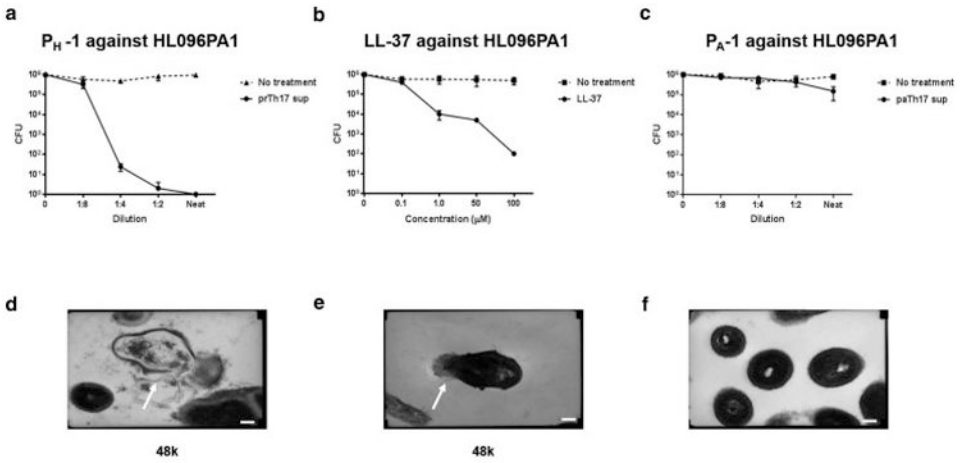

Fig. 5. Supernatants derived from activated prTh17 clones are bactericidal against *P. acnes*

CFU assay results for *P. acnes* strain HL096PA1 after 3 h incubation with **(a)** prTh17 clone P_H-1 supernatants, (**b**) LL-37 (0.1-100μm), and (**c**) paTh17 clone P_A-1 supernatants. Data shows average CFU from three independent experiments (n=3), error bars are mean ± SD. (**d-f**) Representative transmission electron microscopy (TEM) micrographs of (**d**) *P. acnes* strain HL096PA1 after 3 h incubation with activated clone P_H-1supernatants, (**e**) LL-37, and (**f**) activated clone P_A-1supernatants. TEM were imaged at 48K. Compared with the activated P_A-1 supernatants (1:2 dilution and neat), the bacteria exposed to activated P_H-1 supernatants (1:2 dilution and neat) and LL-37 (50μM and 100μM) exhibit cellular differences indicative of stresses on the membrane. Scale bar = 0.5μm.