



Published in final edited form as:

J Invest Dermatol. 2014 February ; 134(2): 366–373. doi:10.1038/jid.2013.334.

***Propionibacterium acnes* induces an interleukin-17 response in acne vulgaris that is regulated by vitamin A and vitamin D**

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Abstract

Acne vulgaris is the most common skin disorder affecting millions of people worldwide and inflammation resulting from the immune response targeting *Propionibacterium acnes* plays a significant role in its pathogenesis. In this study, we have demonstrated that *P. acnes* is a potent inducer of Th17 and Th1, but not Th2 responses in human PBMCs. *P. acnes* stimulated expression of key Th17-related genes, including IL-17A, ROR α , RORc, IL-17RA and IL-17RC, and triggered IL-17 secretion from CD4⁺, but not CD8⁺ T cells. Supernatants from *P. acnes*-stimulated PBMCs were sufficient to promote the differentiation of naïve CD4⁺CD45RA T cells into Th17 cells. Furthermore, we found that the combination of IL-1 β , IL-6 and TGF- β neutralizing antibodies completely inhibited *P. acnes*-induced IL-17 production. Importantly, we showed that IL-17-expressing cells were present in skin biopsies from acne patients but not from normal donors. Finally, vitamin A (all-trans retinoic acid) and vitamin D (1,25-dihydroxyvitamin D3) inhibited *P. acnes*-induced Th17 differentiation. Together, our data demonstrate that IL-17 is induced by *P. acnes* and expressed in acne lesions and that both vitamin A and vitamin D could be effective tools to modulate Th17-mediated diseases such as acne.

Introduction

Acne vulgaris is a multifactorial chronic disorder of the pilosebaceous follicles of human skin and its pathogenesis is not yet completely understood (Kim, 2005; Zouboulis, 2001;

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Conflicts of Interest

The authors state no conflict of interest.

Zouboulis *et al.*, 2005). The inflammatory nature of acne has been associated with the host immune response targeting *Propionibacterium acnes*, a commensal human skin bacterium found in the pilosebaceous follicles.

The innate immune system recognizes *P. acnes* via Toll-like receptor 2 (TLR2) (Kim *et al.*, 2002), leading to the secretion of inflammatory cytokines, including IL-8 and IL-12. *P. acnes* has also been shown to stimulate production of inflammatory cytokines such as IL-8, TNF- α , IL-1 β by both human monocytic cell lines and freshly isolated PBMCs from acne patients and normal controls (Vowels *et al.*, 1995). The adaptive immune response system also plays a central role in the inflammation observed in acne, resulting from the recruitment of activated T helper 1 (Th1) lymphocytes to early acne lesions (Mouser *et al.*, 2003). While the function of innate immune cells and Th1 cells in acne inflammation have been studied, the role of Th17 cells in acne is yet to be elucidated.

Th17 cells are characterized by the production of IL-17A and IL-17F belonging to the IL-17 family of cytokines. IL-17A and IL-17F target mostly nonlymphoid cells, including fibroblasts, keratinocytes, endothelial cells and macrophages and induce the production of cytokines such as TNF- α , IL-6, GM-CSF, and matrix metalloproteinases (Damsker *et al.*, 2010; Kolls and Linden, 2004). In addition to IL-17A and F, Th17 cells produce IL-6, IL-21 and IL-22 and exhibit effector functions distinct from the Th1 and Th2 cells (Harrington *et al.*, 2005; Langrish *et al.*, 2005; Liang *et al.*, 2006; Nurieva *et al.*, 2007; Park *et al.*, 2005; Zheng *et al.*, 2007; Zhou *et al.*, 2007). An important outcome of these effects is localized chronic tissue inflammation, which is often associated with extracellular matrix destruction (Miossec, 2003).

Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many autoimmune disorders such as psoriasis, rheumatoid arthritis, Crohn's disease and multiple sclerosis (Amadi-Obi *et al.*, 2007; Lock *et al.*, 2002; Teunissen *et al.*, 1998). Immune modulators such as ATRA (All-trans retinoic acid) and 1,25D3 (1,25-dihydroxyvitamin D3) share the retinoid X receptor (RXR) as a common receptor for retinoid signaling and have been tried as potential therapeutic agents in a variety of autoimmune and inflammatory diseases (Mucida *et al.*, 2007).

Herein, we have evaluated whether Th17 cells are involved in the inflammatory response towards *P. acnes* and explored a possible role for vitamin A (ATRA) and vitamin D (1,25D3) in modulating Th17 differentiation.

Results

***P. acnes* lab strain and clinical isolates stimulate production of IL-17 and IL-22**

Increased IL-17 production is observed in response to pathogenic microbes and in inflammatory skin conditions such as psoriasis (Hino *et al.*, 2011; Infante-Duarte *et al.*, 2000; Tokura *et al.*, 2010). We sought to determine if the causative pathogen of acne vulgaris, *P. acnes*, could stimulate the production of IL-17 in human peripheral blood mononuclear cells (PBMCs). We observed that both live *P. acnes* and *P. acnes* sonicate (ATCC strain 6919) stimulated the production of IL-17 (Fig. 1a), which was optimally

induced seven days after activation. Other cutaneous pathogens, including *Mycobacterium tuberculosis* and *Mycobacterium leprae*, showed significantly lower IL-17 induction in comparison to *P. acnes*. Meanwhile, *Staphylococcal enterotoxin B* (SEB) was a potent inducer of IL-17, corroborating a previous study (Islander *et al.*, 2010). In addition, we tested seven *P. acnes* clinical isolates obtained from acne patients and found that all clinical isolates tested induced IL-17 protein secretion ranging from approximately 500–700 pg/ml (Fig. 1b; $p < 0.001$). Stimulation of PBMCs with both the *P. acnes* lab strain and clinical isolates also mediated robust IL-22 protein secretion (supplementary Fig. S1a and S1b; $P < 0.001$).

***P. acnes* triggers a Th17 and Th1 response**

Infectious and inflammatory diseases are commonly characterized as Th1, Th2 or Th17, based on the subsets of T cells involved in host defense or disease pathogenesis. Therefore, we next wanted to characterize the T cells resulting from *P. acnes* stimulation based on their ability to produce IFN- γ (Th1), IL-4 (Th2), and/or IL-17 (Th17). We found that *P. acnes* induced IL-17 and IFN- γ but not IL-4 (Fig. 2a). In addition, we found that all seven *P. acnes* clinical isolates induced significant levels of IL-17 and IFN- γ protein expression, but minimal levels of IL-4, as measured by both ELISA (Fig. 2b; $p < 0.001$) and flow cytometry (Fig. 2c). Our data suggest that *P. acnes* induces both Th17 and Th1 immune responses as measured by IL-17 and IFN- γ , respectively.

We next characterized the IL-17 and IFN- γ -expressing T cells based on CD4 and CD8 expression. *P. acnes* induced both IL-17 and IFN- γ in CD4⁺ T cells (Fig. 2d). On the other hand, CD8⁺ T cells produced only IFN- γ but not IL-17, suggesting that the production of IL-17 was restricted to CD4⁺ T cells in response to *P. acnes*.

***P. acnes* induces Th17-related genes**

The hallmark of human Th17 cells is the expression of IL-17 and key differentiation markers including *ROR α* and *ROR γ* . *P. acnes* induced IL-17 gene expression (28-fold), as well as expression of retinoic orphan receptors *Rora* (10-fold) and *Rorc* (14-fold). PBMCs stimulated with *P. acnes* also were induced to express IL-17RA (7-fold) and IL-17RC (8-fold), two receptor genes that partner together to mediate responses to IL-17A and IL-17F (Toy *et al.*, 2006) (Fig. 3; $p < 0.001$). Our data suggest that *P. acnes* triggers the expression of key genes involved in Th17 differentiation, including IL-17, IL-17 receptor genes and the transcriptional factors *Rorc* and *Rora*.

Supernatants from PBMCs treated with *P. acnes* differentiate naïve CD4⁺CD45RA T cells to IL-17 producing Th17 cells

The differentiation of Th17 cells occurs in the presence of specific cytokines including IL-1 β , IL-6 and TGF- β . We hypothesized that culture supernatants from *P. acnes*-stimulated PBMCs were sufficient to induce naïve CD4⁺CD45RA T cells to differentiate into Th17 cells. We isolated naïve CD4⁺CD45RA T cells from the peripheral blood of healthy donors and stimulated them with plate-bound anti-CD3 and soluble anti-CD28 monoclonal antibodies for six days in the presence or absence of culture supernatants derived from *P. acnes*-stimulated PBMC. Those naïve T cells activated in the presence of supernatants from

P. acnes-stimulated PBMCs produced IL-17, with the frequency of IL-17 secreting cells increasing from 0.01 to 1.4% (Fig. 4a). We also observed differentiation of naïve T cells into Th1 cells as measured by IFN- γ (4.25 %), but not Th2 cells, as measured by IL-4 (Supp Fig S2). Treatment with anti-CD3 alone did not facilitate differentiation into IL-17 producing cells (Supplementary Fig S2). These data indicate that activation of PBMCs with *P. acnes* triggers the secretion of cytokines that promote the differentiation of naïve T cells into Th1 and Th17 cells.

To determine the key cytokines involved in the development and differentiation of *P. acnes*-induced IL-17-expressing T cells, we incubated PBMCs for one hour with neutralizing antibodies to IL-17, IL-1 β , IL-6, TGF- β , IL-23p19, IL-4 and IFN- γ prior to treatment with *P. acnes*. The cells were cultured and evaluated for cytokine expression by ELISA on day seven. *P. acnes* induced the production of IL-17 which could be inhibited in the presence of neutralizing antibody to IL-17. The addition of IL-1 β , IL-6, IL-23p19 or TGF- β neutralizing antibody alone led to the reduction of IL-17 protein expression by approximately 40–50% ($p < 0.001$) (Fig. 4b). In addition, the combination of all four antibodies completely abrogated *P. acnes*-induced IL-17 production ($p < 0.001$). In contrast, neutralization of IL-23p19, IL-4 or IFN- γ alone had no effect on IL-17 production. No significant change in IL-17 production was found in the presence of isotype control antibodies (data not shown). Finally, differentiation of naïve T cell into Th1 cells as measured by IFN- γ production was blocked by neutralizing IL-12p40; in contrast, the presence of the Th17 associated neutralizing antibodies had no effect (Supplementary Fig S2b). Therefore, IL-1 β , IL-6 and TGF- β appear to regulate *P. acnes*-induced IL-17 responses and corroborate previous studies demonstrating that these cytokines are important for the generation and maintenance of Th17 T cells.

IL-17-expressing cells are found in acne skin lesions

To date there are no studies clearly linking IL-17 to the pathogenesis of acne. Given our data that *P. acnes* induces a Th17 response *in vitro*, we sought to correlate these findings with *in situ* studies of IL-17 expression in skin biopsies from acne patients and healthy individuals. We found that IL-17-expressing cells were present in skin biopsies of acne patients and scattered around the dermis near the pilosebaceous unit (Fig. 5). On the other hand, IL-17 expression was not detectable in skin biopsies from healthy donors and isotype controls. Taken together, our immunohistochemistry results demonstrate that cells expressing IL-17 are detected in acne skin biopsies.

ATRA and 1,25D3 inhibit the development of Th17 cells

Vitamin A (ATRA) and vitamin D (1,25D3) share retinoid X receptor (RXR) as a common receptor for signaling, and their functions on inhibiting Th17 development are thought to be similar (Iwata *et al.*, 2003; Sigmundsdottir *et al.*, 2007). ATRA and 1,25D3 are commonly used dermatologic therapeutics as they have the ability to modulate the immune response. We therefore compared the role of ATRA and 1,25D3 on *P. acnes*-induced Th17 generation. *P. acnes* stimulation in the absence of ATRA and 1,25D3 (10^{-7} M) induced expression of IL-17 mRNA and protein expression (Fig. 6a; $p < 0.001$, 6b; $p < 0.001$). The addition of either ATRA, 1,25D3, or a combination of the two prior to activation with *P. acnes* downregulated

the induction of both *IL-17* mRNA and protein expression (Fig. 6a, 6b); the inactive form of vitamin D3 (25D3) had no effect. Similar results were obtained for the expression of *RORa* and *RORc* genes (Fig. 6c and 6d; $p < 0.001$); both ATRA and 1,25D3 downregulated the induction of *RORa* (74 and 83%) and *RORc* (73 and 80%) expression, respectively. The combination of ATRA and 1,25D3 strongly inhibited *RORa* and *RORc* expression by 83% and 93%, respectively. In contrast, neither ATRA nor 1,25D3 had any significant inhibitory effect on IL-17RA and IL-17RC receptor gene expression (Fig. 6e, 6f; $p < 0.001$). Finally, we determined that ATRA and 1,25D3 did not have a significant affect on cell viability (Supplementary Fig. S3). Our findings strongly suggest that both ATRA and 1,25D3 effectively inhibit the expression of genes required for the development and differentiation of *P. acnes*-related Th17 cells.

Discussion

IL-17 is a cytokine secreted by activated T cells and has been implicated in the pathogenesis of both infectious and inflammatory skin diseases such as Staphylococcus infection, psoriasis, contact hypersensitivity and atopic dermatitis (Cho *et al.*, 2010; Guttman-Yassky *et al.*, 2008; Hino *et al.*, 2011; Pennino *et al.*, 2010; Tokura *et al.*, 2010). While inflammation induced by both innate and adaptive immune responses in acne has been studied, the role of IL-17 producing Th17 cells in acne pathogenesis has not been investigated. Here, we demonstrate the involvement of IL-17 in acne pathogenesis to our knowledge previously unreported. *P. acnes* stimulation of PBMCs induced production of IL-17 and IL-22, as well as the expression of key Th17 differentiation markers including ROR α , ROR γ , and IL-17 receptors, IL-17RA and IL-17RC. Antibody neutralizing experiments demonstrated that IL-1 β , IL-6 and TGF- β were key inducers of Th17 cell activation. Importantly, we described IL-17-expressing cells surrounding the inflamed pilosebaceous unit in skin biopsies from acne patients. Finally, ATRA and 1,25D3, two immunomodulators used for treatment of various skin diseases, inhibited the development of Th17 cells and IL-17 production. Together our data suggest that Th17 cells may play a role in the pathogenesis of acne.

Infectious and inflammatory diseases have been categorized based on the T cells and cytokines that characterize the diseased tissue. For example, a Th1 response predominates in psoriasis, while a Th2 response predominates in atopic dermatitis. In leprosy, a disease caused by *Mycobacterium leprae*, the protected tuberculoid patients mount predominantly a Th1 response while the susceptible individuals mount a Th2 response (Modlin, 1994). We found that *P. acnes* induced IFN- γ but not IL-4, similar to a previous study (Mouser *et al.* 2004). However, we also showed that IL-17 was induced indicating that both Th1 and Th17 responses may be involved in the host response to *P. acnes*. Several infectious and inflammatory diseases involve both Th1 and Th17 response such as psoriasis, which initially was shown to be Th1-mediated, but recent work demonstrates the involvement of IL-17 (Kagami *et al.*, 2010). Therefore, similar to psoriasis, treatments targeting both Th1 and Th17 cytokines in acne may be therapeutic. Interestingly, we found IL-17-expressing cells surrounding the pilosebaceous unit in acne lesion suggesting that our *in vitro* data is clinically relevant. It would be useful to investigate whether the levels of response of Th1 and Th17 and even Th2 might vary in various acne lesions, similar to the immune spectrum

seen in leprosy patients, particularly given that *Mycobacterium* and *P. acnes* share similar genetic features (Barksdale and Kim, 1984).

We determined that distinct subsets of T cells were responsible for the secretion of IL-17 and IFN- γ in response to *P. acnes*. *P. acnes* induced both IL-17 and IFN- γ in CD4⁺ T cells, but not CD8⁺ T cells, which produced only IFN- γ . Our data support previous studies that suggest that inflammation in acne can be initiated by a *P. acnes*-specific CD4⁺ T cell response (Farrar and Ingham, 2004; Lodes *et al.*, 2006; Teunissen *et al.*, 1998). Indeed, it has been postulated that immunogenic *P. acnes* proteins released into the follicle could be processed by Langerhans cells, which could, in turn present these antigens to CD4⁺ T cells in local lymph nodes (Farrar and Ingham, 2004). Whether the Th17 cells found in the periphery migrate back to skin to induce subsequent inflammation in acne resulting in inflammation is not clear.

Our data identified three cytokines, IL-1 β , IL-6 and TGF- β to be critical for triggering the differentiation programs of naïve CD4⁺ T cells to IL-17 secreting Th17 cells in response to *P. acnes*. In contrast, neither IL-23, IFN- γ or IL-4 was required for Th17 differentiation *in vitro*. This finding corroborate other studies showing the combinatorial role of IL-1 β , IL-6 and TGF- β in the initiation of Th17 cell development (Acosta-Rodriguez *et al.*, 2007; Korn *et al.*, 2007; Li *et al.*, 2007; Mangan *et al.*, 2006; Sutton *et al.*, 2006; Wilson *et al.*, 2007; Zhou *et al.*, 2007). The fact that we detected IL-17 differentiation in the absence of IL-23 suggests that IL-23 may be dispensable in the induction of *P. acnes*-specific IL-17 response. However, we do not exclude the possibility that IL-23 and other cytokines such as IL-21 and IL-22 may support the later stages of Th17 differentiation.

Finally, we examined the role of vitamin A and vitamin D in regulating the generation of Th17 cells. Both ATRA and 1,25D3 have has been shown to suppress Th17 generation via downregulation of RAR α and the transcriptional factor ROR γ c, which orchestrates Th17 differentiation (Chang *et al.*, 2010; Ikeda *et al.*, 2010; Mucida *et al.*, 2007). We found that ATRA and 1,25D3 inhibited IL-17 mRNA and protein expression in response to stimulation with *P. acnes*. In addition, induction of *RORa* and *RORc* was also inhibited in the presence of ATRA or 1,25D3. On the other hand, ATRA and 1,25D3 had no significant effect on IL-17 receptor gene expression. These findings suggest a role for retinoids and vitamin D in regulating the expression of genes required for Th17 differentiation.

In summary, we demonstrate that, *P. acnes* is a potent inducer of IL-17, IL-22 and IL-17-associated genes in human PBMCs and that IL-17 producing cells are found in acne lesions. Our *in vivo* finding that IL-17⁺ lymphocytes are present in abundance near the pilosebaceous follicles suggests that Th17 cells may play a role in acne pathogenesis. In addition, we demonstrate the inhibitory effect of ATRA and 1,25D3 in *P. acnes* induction of IL-17, providing a therapeutic application of ATRA and 1,25D3 for the treatment of acne and other Th17 mediated skin disease.

Materials and Methods

PBMC isolation, stimulation and cytokine ELISAs

PBMCs were obtained from healthy donors after signed written informed consent as approved by the Institutional Review Board at UCLA in accordance with the Helsinki Guidelines. PBMCs were then isolated using Ficoll–Paque gradients (GE Healthcare) and plated onto 24 well tissue culture plates (2×10^6 to 5×10^6 /well) in RPMI 1640 media containing 10% FBS (HyClone). Cells were cultured with media, *P. acnes* sonicate (2 µg/ml), live *P. acnes* 0.5 multiplicity of infection (0.5 MOI), *Mycobacterium tuberculosis* sonicate (2 µg/ml), *Staphylococcus enterotoxin B* (2 µg/ml) or *Mycobacterium leprae* sonicate (2 µg/ml) for 7 days. IL-17 and IL-22 levels in culture supernatants were measured by ELISA following the manufacturer's recommendations (R&D ELISA Development System, DY317 and DY782). Samples were assayed in triplicates.

P. acnes and Clinical Isolates

P. acnes strain ATCC 6919 was obtained from American Type Culture Collections and prepared by probe sonication. The level of endotoxin contaminating the *P. acnes* was quantified with a *Limulus* Amoebocyte Lysate assay (BioWhittaker) and found to be < 0.1 ng/ml. *P. acnes* cultures were also grown in the miniMACS anaerobic workstation (Don Whitley Scientific) as described before (Marinelli *et al.*, 2012).

P. acnes clinical isolates were obtained from nasal skin microcomedones from patients attending the Division of Dermatology outpatient clinic at UCLA, or from donors recruited to the laboratory as previously described (Marinelli *et al.*, 2012) and after signed written informed consent as approved by the Institutional Review Board at UCLA in accordance with the Helsinki Guidelines.

Surface and Intracellular staining

Cells were collected and left unstimulated or were stimulated with *P. acnes* in the presence of GolgiStop (BD) for the last five hours of incubation prior to staining. As a positive control for IL-17 cytokine secretion, cells were incubated for five hours with 50 ng/ml of Phorbol 12-Myristate 13-acetate, 500 ng/ml Ionomycin (Sigma), and Golgiplug (IFN- γ and IL-17) or GolgiStop (IL-17 and IL-4) at the recommended concentrations (BD Pharmingen). Cells were first surface stained with fluorescein isothiocyanateconjugated anti-CD4 or anti-CD8 antibodies (BD) for 20 minutes on ice. Intracellular staining was performed using the Cytotfix-Cytosperm buffer set following manufacturer's protocol (BD). Cells were fixed with 2% paraformaldehyde, acquired on BD Biosciences FACSscan, and analyzed using CellQuest-Pro software (BD).

Purification and stimulation of naïve CD4⁺ T cells

Naïve CD4⁺ T cells were isolated by negative selection using the EasySep Human Naïve CD4⁺ T Cell Enrichment Kit (Stem Cell Technologies) following manufacturer's recommendations. PBMCs were isolated and stimulated with media, live *P. acnes* (0.5 MOI) or *P. acnes* sonicate at 2 µg/ml for 24 hours. Supernatants were harvested after 24 hours and added to cultures containing naïve CD4⁺ T cells that had been stimulated with plate bound

anti-human CD3 and anti-human CD28 (eBiosciences) antibodies. Intracellular cytokine staining was performed on day seven.

RNA isolation, cDNA synthesis and real-time PCR

PBMCs were stimulated with media or *P. acnes* sonicate. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocol and treated with RNase-free DNase. RNA samples were reverse-transcribed to cDNA using iScript cDNA synthesis kit (BIO-RAD). Reactions were done at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. Real-time PCR was applied using iQ SYBR Green supermix (BIO-RAD). 40 cycles were carried out at 95°C for 5 min, then 95°C for 10 sec, 55°C for 20 sec, 72°C for 20 sec. *GAPDH* was used as a control. Gene expression level was quantified by the comparative method 2^{-CT} . The list of primers used in the study are summarized in supplementary table S1.

Cytokine blocking/Neutralization experiments

PBMCs were incubated with neutralizing antibodies for one hour prior to activation with *P. acnes* sonicate. The following antibodies were used: anti-IL-1 β (1 μ g/ml; R&D systems), anti-IL-17 (2 μ g/ml; R&D systems), anti-IL-23 p19 (0.4 μ g/ml; R&D systems), anti-TGF- β (1 μ g/ml; R&D systems), anti-IL-4 (100 ng/ml; BD Pharmingen), anti-IL-6 (300 ng/ml; BD Pharmingen), and anti-IFN- γ (300 ng/ml; BD Pharmingen), anti IL-12p40/70 (300 ng/ml; BD Pharmingen). Samples were assayed in triplicates.

Immunohistochemistry

De-identified normal skin biopsy specimens obtained from the UCLA Translational Pathology Core Laboratory and typical closed comedone-type acne lesions were obtained from Dankook University Hospital after signed written informed consent of patients as approved by the Institutional Review Board in accordance with the Helsinki Guidelines for protection of human subjects. Immunohistochemical analysis was performed using the standard streptavidin–biotin technique, using the commercial kit HRP-AEC system following manufacturer's recommendations (R&D Systems).

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

Cell proliferation experiments were performed in 96-well plates (5 replicates) and 1,25D3 (10^{-7} M) and ATRA (10^{-7} M) treatments initiated at 24 hours post-seeding for 7 days. Stocks and dilutions of PLX4032/vemurafenib (Plexxikon) and AZD6244/selumetinib (Selleck Chemicals) were made in dimethyl sulfoxide (DMSO). Cells were quantified using CellTiter-GLO Luminescence (Promega) following the manufacturer's recommendations

Statistical analysis

Experimental values were expressed as the means \pm standard deviation (SD) for the number of separate experiments indicated in each case. To assess differences in expression level for all groups a generalized estimating equation (GEE) model was run because donors were repeatedly measured over time. Overall group F tests were performed and if significant, post hoc pairwise comparisons were run between the groups using Tukey's multiple comparison

criteria to control the familywise error rates. Both Tukey intervals and Tukey adjusted p-values were used for this analysis. Significant differences were considered for those probabilities < 5% ($p < 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Ping Fu (Tissue procurement and Histology Core Laboratory, UCLA) for her assistance with histological preparations. Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Centers for AIDS Research Flow Cytometry Core Facility supported by NIH Awards CA-16042 and AI-28697, by the JCCC, the UCLA AIDS Institute and the UCLA School of Medicine. This work was supported by NIH Grant R01-AR-053542 (J.K.). Statistical analysis was funded by the UCLA Clinical and Translational Science Institute Grant UL1TR00124

Abbreviations

ATRA	All-trans retinoic acid
IL-17	Interleukin 17
PBMCs	Peripheral blood mononuclear cells
Th17	T helper 17
TGF-β	Transforming growth factor- β
GM-CSF	Granulocyte-macrophage colony stimulating factor
RORC	Retinoic orphan receptor C
RORA	Retinoic orphan receptor A
1,25D3	1,25-dihydroxyvitamin D3

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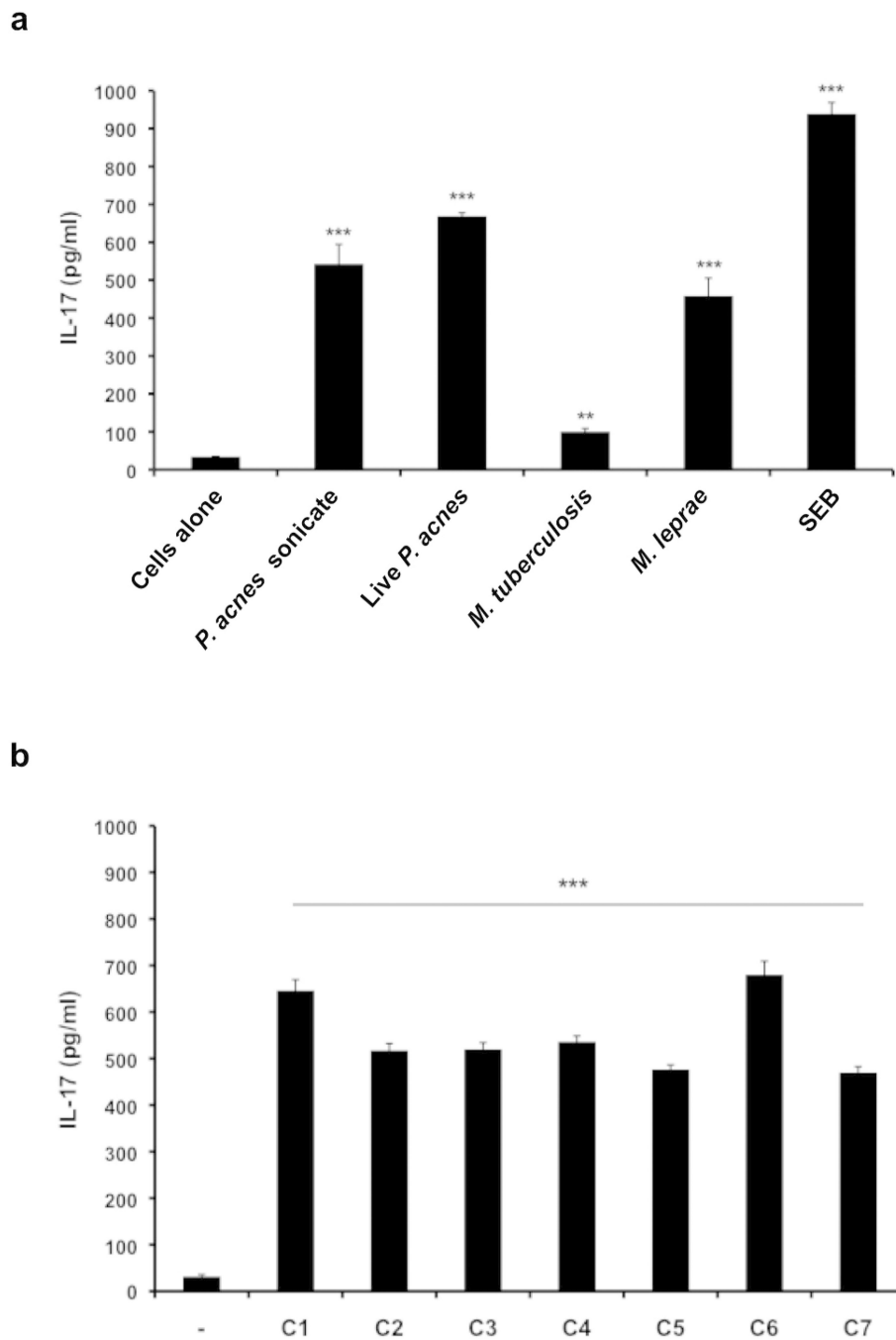


Fig.1. *P. acnes* lab strain and clinical isolates stimulate production of IL-17 in human PBMCs
a) PBMCs were cultured ($2-5 \times 10^6/ml$) in the presence of *P. acnes* sonicate ($2 \mu g/ml$), live *P. acnes* (0.5 multiplicity of infection), *M. tuberculosis* ($5 \mu g/ml$), *M. leprae* ($5 \mu g/ml$), and *Staphylococcus* enterotoxin B (SEB $2 \mu g/ml$) for seven days. **b)** PBMCs ($2-5 \times 10^6/ml$) were cultured either in the presence or absence of seven *P. acnes* clinical isolates (C1–C7). Levels of IL-17 accumulated in culture supernatants were measured using ELISA. Experiments were performed at least five times using PBMCs from five different donors with similar results. (** p 0.05, ***p 0.001)

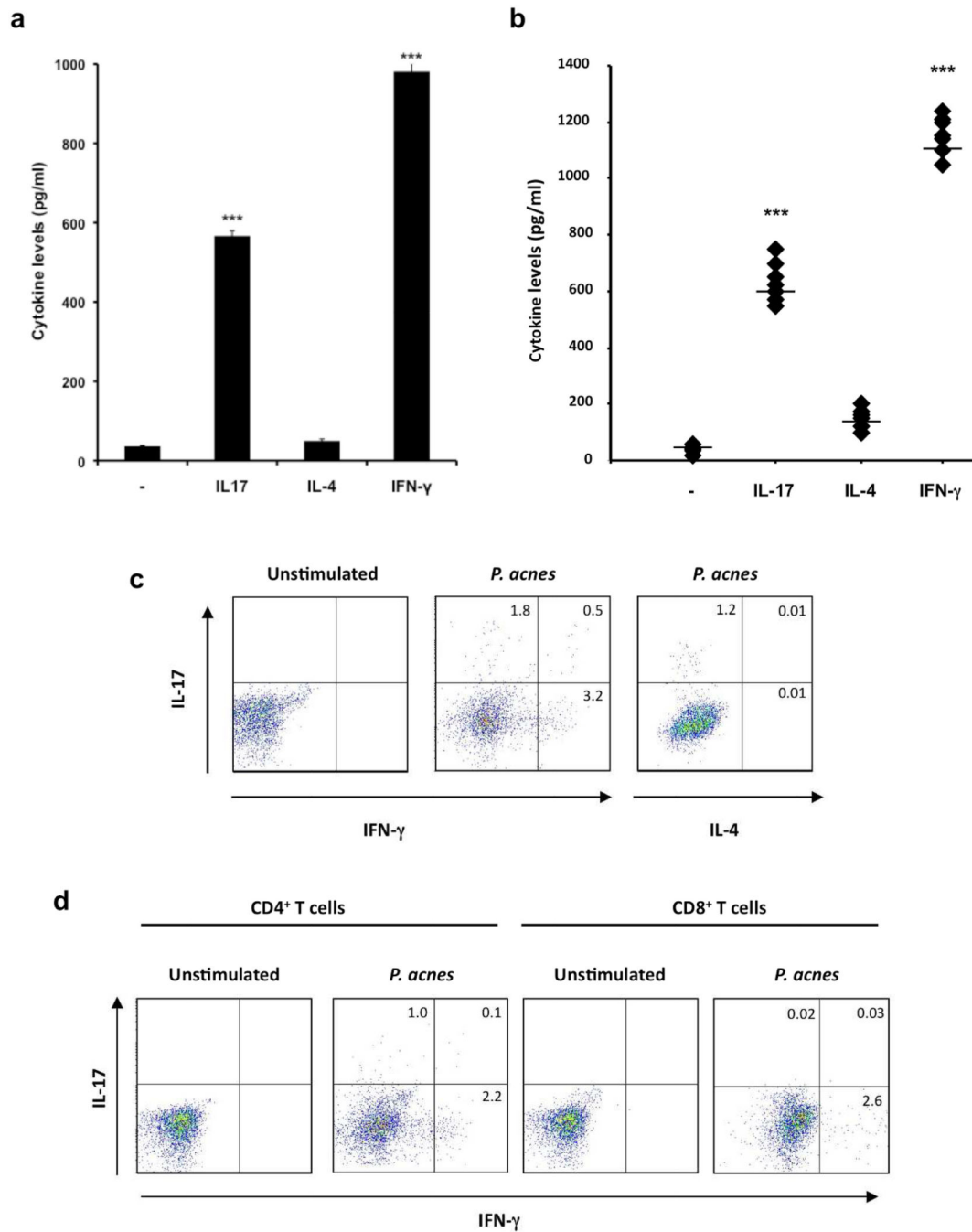


Fig.2. *P. acnes* stimulate production of IL-17A and IFN-γ but not IL-4 in PBMCs
 PBMCs were cultured ($2-5 \times 10^6$ /ml) in the presence of *P. acnes* sonicate ($2 \mu\text{g/ml}$) or *P. acnes* clinical isolates for 7 days. **a)** Levels of IL-17, IL-4 and IFN- γ accumulated in culture supernatants were measured using ELISA. Experiments were performed at least five times using PBMCs from five different donors with similar results. **b)** PBMCs ($2-5 \times 10^6$ /ml) were cultured either in the presence or absence of seven *P. acnes* clinical isolates (C1-C7). Levels of IL-17, IL-4, and IFN- γ accumulated in culture supernatants were measured using ELISA. Experiments were performed at least three times using PBMCs from three different

donors with similar results. The overall group effect was statistically significant ($p < 0.001$).

c) Flow cytometry of PBMCs stimulated with *P. acnes* sonicate for 7 days. Intracellular cytokine staining for IFN- γ , IL-4 and IL-17 was performed on day 7. Plots are gated on CD4⁺ T cells. Each panel is representative of four independent experiments. **d)** Flow cytometry of peripheral blood stimulated with *P. acnes* sonicates for seven days. Intracellular cytokine staining for IFN- γ and IL-17 was performed on day seven. Plots are gated on CD4⁺ and CD8⁺ T cells. Each panel is representative of three independent experiments. Data represent mean \pm SD (** $p < 0.05$, *** $p < 0.001$)

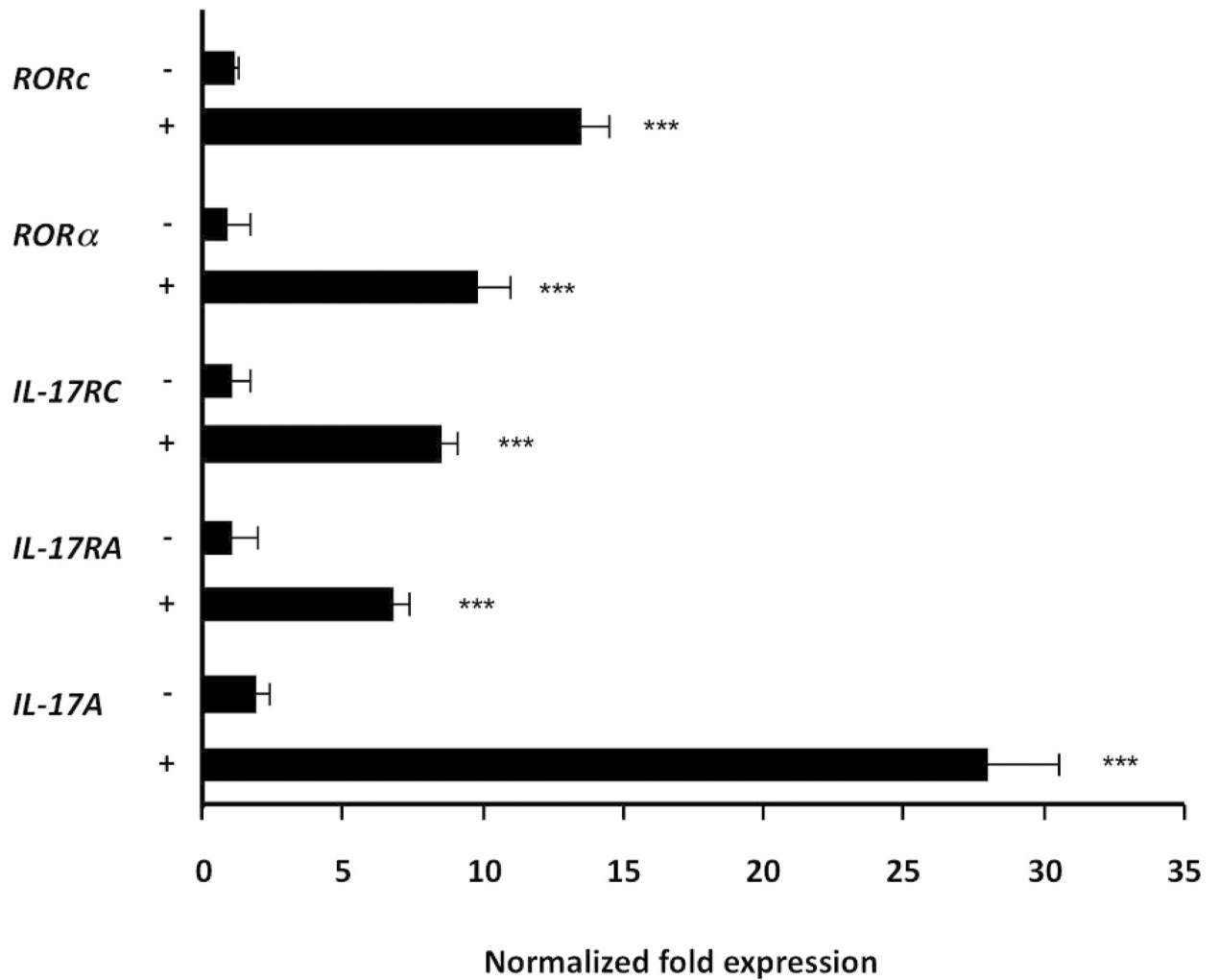
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Fig.3. Induction of *IL-17*, *IL-17RA*, *IL-17RC*, *RORc* and *RORα* mRNAs expression in PBMCs stimulated with *P. acnes*

PBMCs were cultured ($2-5 \times 10^6$ /ml) with *P. acnes* sonicate (2 μ g/ml). Real time PCR of *IL-17*, *IL-17RA*, *IL-17RC*, *RORc* and *RORα* mRNA expression was analyzed 24 hours following *P. acnes* stimulation. Gene expression was normalized to the housekeeping genes *GAPDH* and quantified by the comparative method 2^{-CT} . Data are representative of four independent experiments. Data represent mean \pm SD (**p < 0.01, ***p < 0.001)

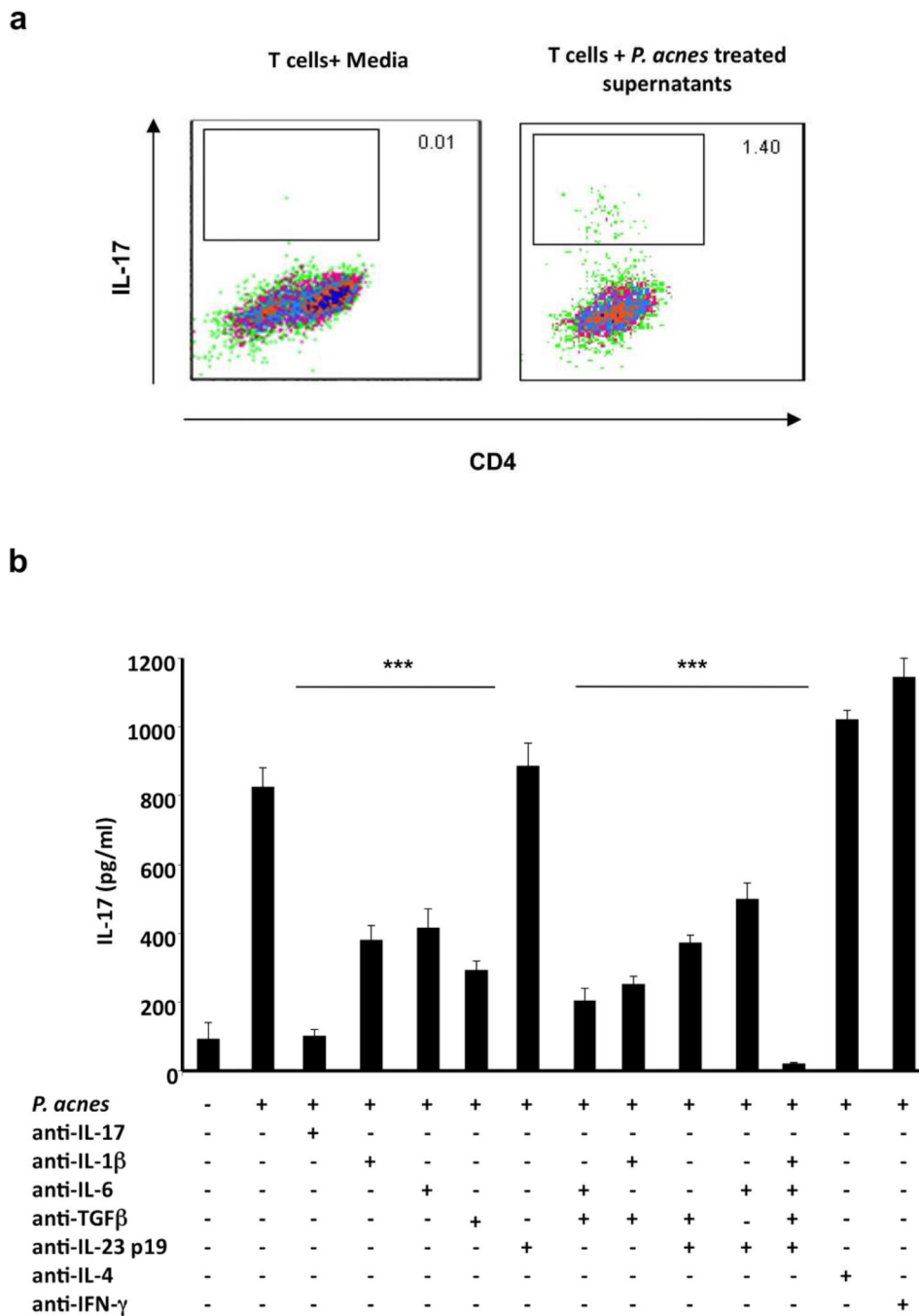


Fig.4. Supernatants from PBMCs treated with *P. acnes* differentiate naïve CD4⁺T cells to IL-17 producing T cells

a) PBMCs ($2-5 \times 10^6/ml$) were stimulated overnight with *P. acnes* sonicate ($2 \mu g/ml$).

Culture supernatants were then collected and used to stimulate naïve CD4⁺CD45RA T cells for seven days in 96 well plates with plate bound anti-CD3 and soluble CD28. Cells were harvested and intracellular cytokine staining for IL-17 was performed. Each panel is representative of three independent experiments. **b)** PBMCs ($2-5 \times 10^6/ml$) were cultured with IL-17, IL-1 β , IL-6, TGF- β , IL-23p19, IL-4 and IFN- γ neutralizing antibodies for one

hour followed by seven days of stimulation with *P. acnes*. IL-17 production was then measured using ELISA. Data are representative of four independent experiments. Data represent mean \pm SD (** p 0.05, ***p 0.001)

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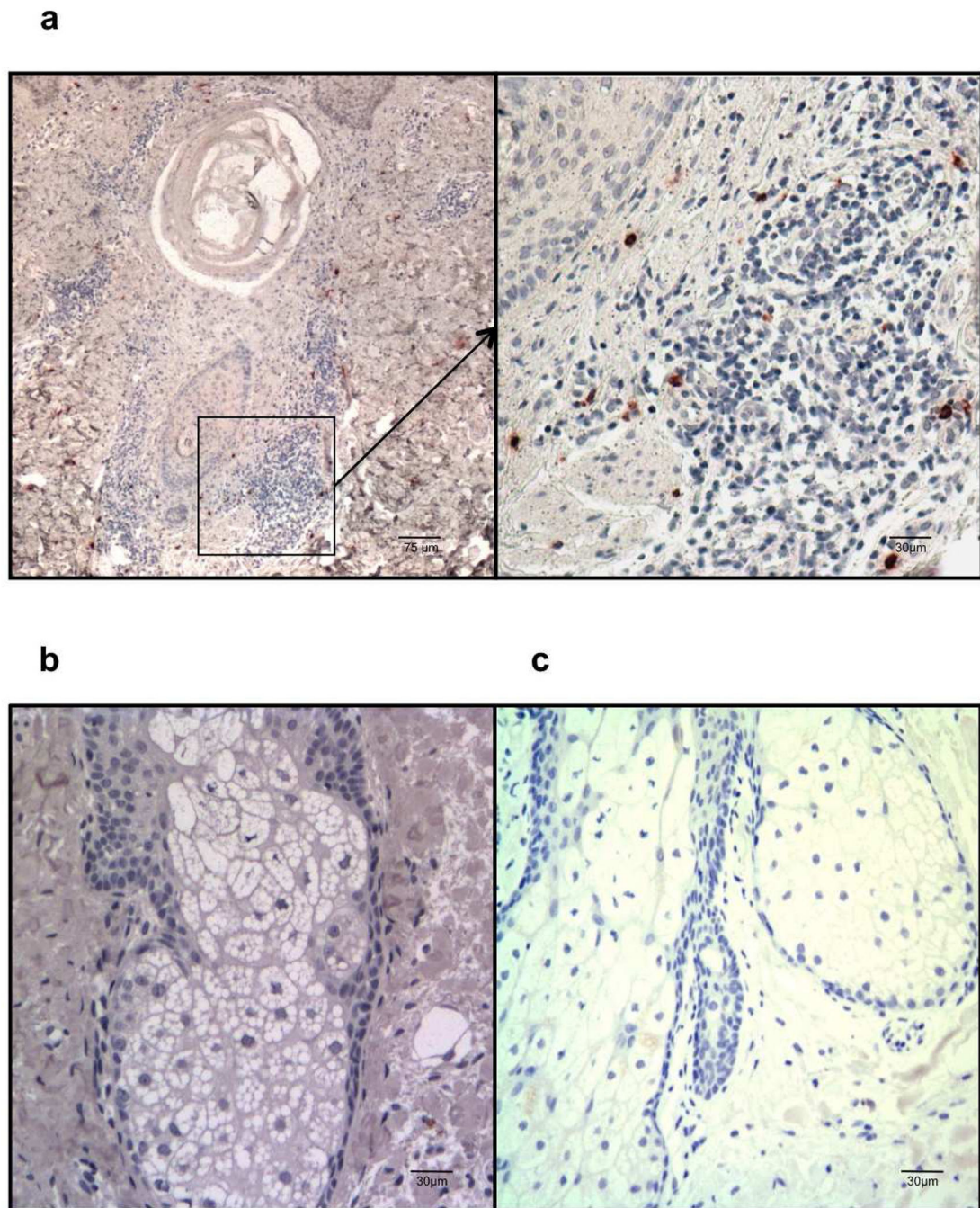


Fig.5. Immunohistochemistry of IL-17 in acne lesion

Frozen skin sections were obtained from patients with acne lesion (a) and (b) and normal control skin (c). Immunohistochemical staining was carried out using anti-human IL-17 antibody (a) and (c) and an isotype control (b). IL-17 expressing lymphoid cells (brown) can be seen scattered around the inflammation surrounding the pilosebaceous follicle (a). Data is representative of three independent experiments from three different lesions. Scale bars represent 75 μm and 30 μm respectively.

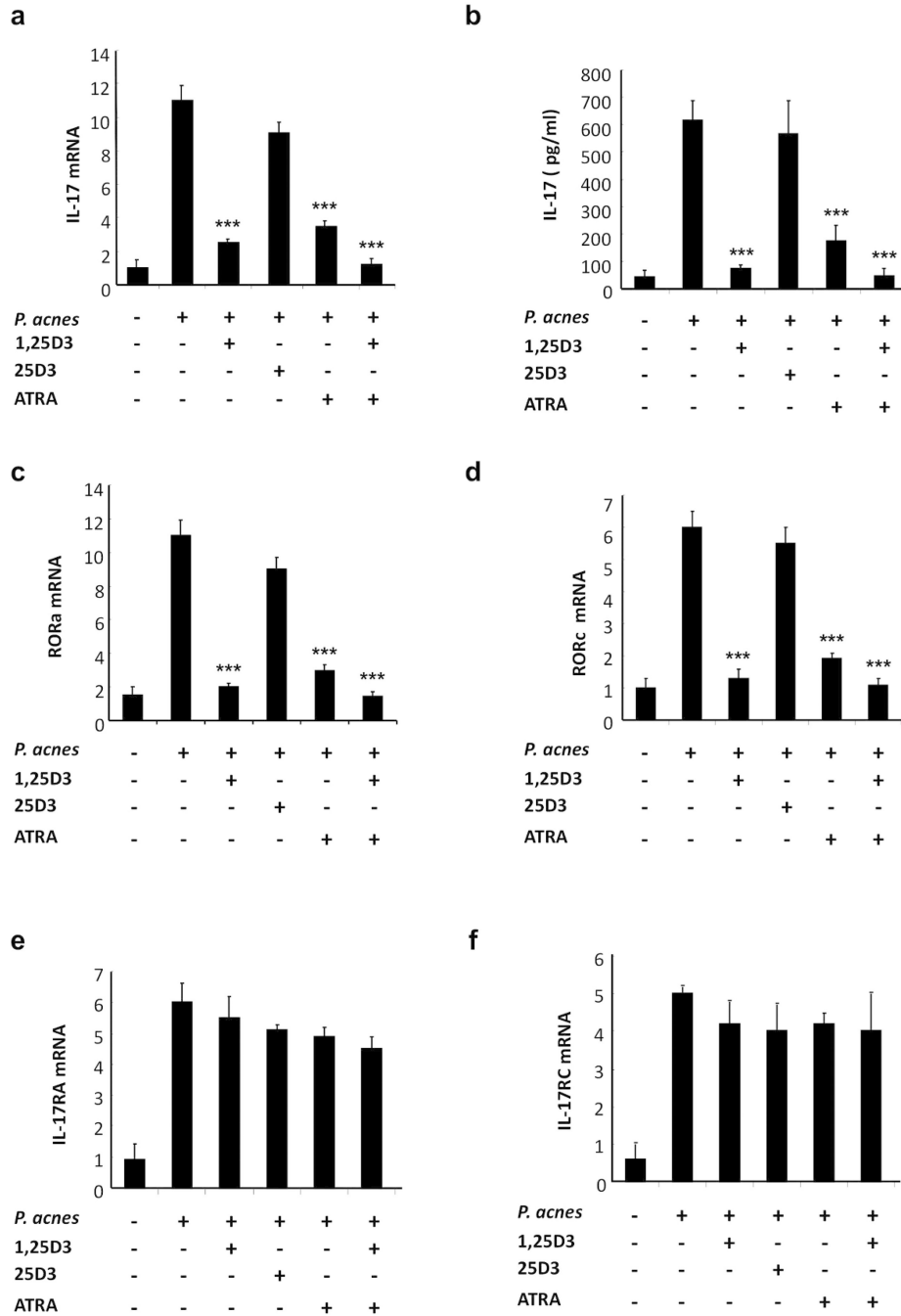


Fig.6. Effects of 1,25D3, 25D3 and ATRA on Th17 differentiation

Human PBMCs were stimulated with *P. acnes* sonicate (2 µg/ml) in the presence of 1, 25D3 (10⁻⁷ M), 25D3 (10⁻⁷ M) and ATRA (10⁻⁷ M). *IL-17* (a), *RORa* (c), *RORc* (d), *IL-17RA* (e) and *IL-17RC* (f) mRNA expression was analyzed 24 hours following *P. acnes* stimulation. Gene expression was normalized to the housekeeping genes *GAPDH* and quantified by the comparative method 2^{-CT}. Each panel is representative of three independent donors. **b)** PBMCs were cultured (2–5 × 10⁶/ml) with 1,25D3, 25D3 and ATRA for one hour followed by seven days of stimulation with *P. acnes* (sonicate or live), IL-17 production was then

measured using ELISA. Data are representative of four independent experiments. Data represent mean \pm SD (**p < 0.001)

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