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Antimicrobial Activity of Sebocytes against Propionibacterium acnes via Toll-Like Receptor 2 and Lysosomal Pathway

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.06.016.

REFERENCES

Campbell P, Morton PE, Takeichi T, Salam A, Roberts N, Proudfoot LE, et al. Epithelial inflammation resulting from an inherited loss-of-function mutation in EGFR. J Invest Dermatol 2014;134:2570–8.

Choate KA, Lu Y, Zhou J, Choi M, Elias PM, Farhi A, et al. Mitotic recombination in patients with ichthyosis causes reversion of dominant mutations in KRT10. Science 2010;330:94–7.

Choate KA, Lu Y, Zhou J, Elias PM, Zaidi S, Paller AS, et al. Frequent somatic reversion of KRT1 mutations in ichthyosis with confetti. J Clin Invest 2015;125:1703–7.

Hotz A, Oji V, Bourrat E, Jonca N, Mazereeuw-Hautier J, Betz RC, et al. Expanding the clinical and genetic spectrum of KRT1, KRT2 and KRT10 mutations in keratinopathic ichthyosis. Acta Derm Venereol 2016;96:473–8.

Knöbel M, O'Toole EA, Smith FJ. Keratins and skin disease. Cell Tissue Res 2015;360:583–9.

Lim YH, Qiu J, Saraceni C, Burrall BA, Choate KA. Genetic reversion via mitotic recombination in ichthyosis with confetti due to a *KRT10* polyalanine frameshift mutation. J Invest Dermatol 2016;136:1725–8.

Oji V, Tadini G, Akiyama M, Blanchet Bardon C, Bodemer C, Bourrat E, et al. Revised nomenclature and classification of inherited ichthyoses: results of the First Ichthyosis Consensus Conference in Sorèze 2009. J Am Acad Dermatol 2010;63:607–41.

Antimicrobial Activity of Sebocytes against *Propionibacterium acnes* via Toll-Like Receptor 2 and Lysosomal Pathway

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TO THE EDITOR

Sebocytes are resident cells of the pilosebaceous unit and are essential to acne pathogenesis through their role in sebum production. Although sebocytes have traditionally been thought of only as lipid-producing cells, recent findings show that sebocytes and other skin-resident cells, such as keratinocytes, also function as innate immune cells, capable of secreting proinflammatory cytokines and releasing antimicrobial peptides (AMPs) in response to bacterial triggers like *Propionibacterium acnes* (Nagy et al., 2005, 2006). *P. acnes* has been shown to exist within the follicular lumen (Kligman, 1974; Knutson, 1974) but has not yet been directly visualized in sebocytes or keratinocytes along the follicle. Nonetheless, *P. acnes*-specific 16s ribosomal RNA has been identified within the follicle and dermis in normal skin (Nakatsuji et al., 2013).

To broaden the understanding of sebocytes as immunocompetent cells, we further investigated and identified new innate immune properties of sebocytes—their ability to uptake *P. acnes* and induce antimicrobial response by two distinct pathways, via the lysosomal pathway and AMP induction. Although there is no clear evidence for *P. acnes* within sebocytes in vivo, the killing of intracellular bacteria by sebocytes could have a role when sebocytes encounter *P. acnes* in the intact or ruptured follicle as part of acne pathogenesis.

Using confocal fluorescence microscopy, we visualized the interaction of cultured sebocytes with *P. acnes* at 0.5 multiplicity of infection, using the immortalized human sebocyte cell line SEB-1. Upon infection, SEB-1 cells were found to internalize *P. acnes* within 30 minutes (Figure 1a). Because *P. acnes* is classified as an extracellular bacteria, its presence within the sebocyte suggests it could be actively taken up by various mechanisms. Further studies are required to understand the specific active processes involved in the uptake of *P. acnes* by sebocytes.

We next assessed the antimicrobial activity of sebocytes infected with *P. acnes* by measuring the number of viable intracellular bacteria using a colony-formation unit assay. Sebocytes were washed three times with phosphate buffered saline to wash away extracellular bacteria before the assay. The percentage of viable *P. acnes* after 24 hours of incubation was 30% of the amount that was initially internalized (Figure 1b), suggesting that SEB-1 could not only internalize *P. acnes* but also trigger a significant antimicrobial response.

Because lysosomal pathways are often used by phagocytes to induce an antimicrobial response (Cooper, 2000), we examined the possibility of a lysosomal pathway mediating antimicrobial activity in sebocytes. First, our confocal microscopy images showed that

Abbreviations: AMP, antimicrobial peptide; hBD2, human beta defensing-2

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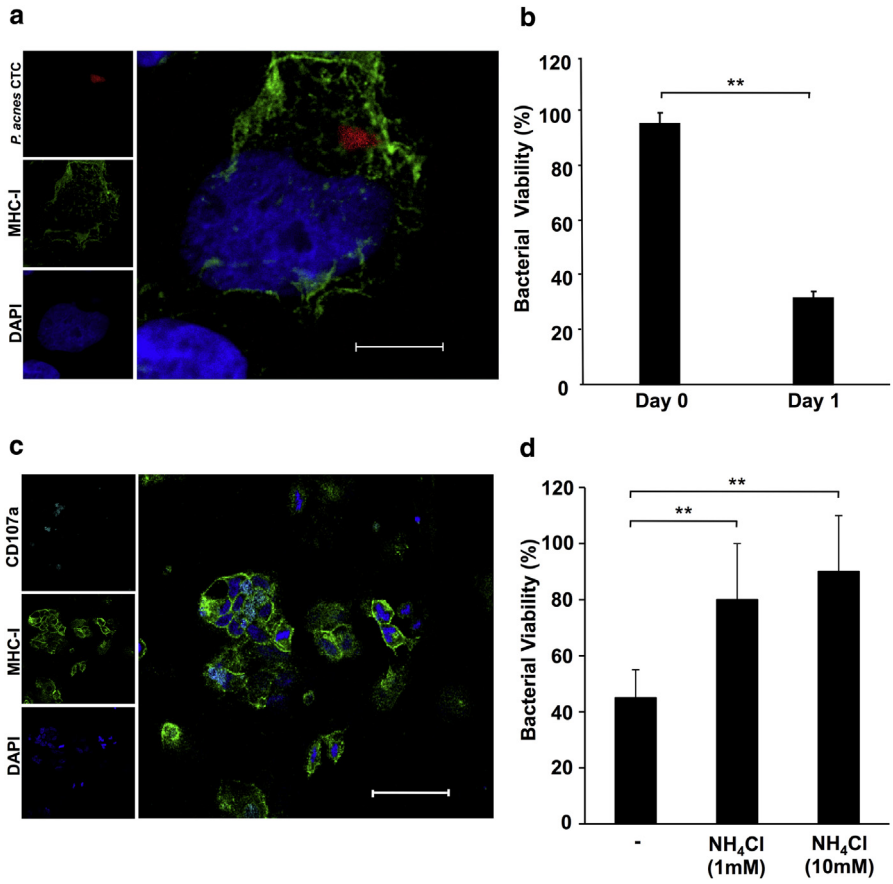

Figure 1. Antimicrobial activity of sebocytes against *P. acnes* and effect of lysosomal pathway inhibition on *P. acnes* viability. (**a**) *P. acnes* (MOI = 0.5) labeled with CTC, a monotetrazolium redox dye that produces a fluorescent formazan upon reduction by live bacterium, was added to cultured SEB-1 cells labeled with MHC class I surface protein-specific antibodies and DAPI and then imaged using confocal fluorescence microscopy after 30 minutes. Scale bar = 10 μm. (**b**) Cells were infected with *P. acnes* (MOI = 5) for 3 hours, washed three times with phosphate buffered saline, counted, and plated. Sebocytes were lysed by resuspension of 0.3% solution of saponin with vigorous pipetting after 45 minutes (day 0) or 24 hours (day 1), and the viable intracellular bacterial load was assessed using CFU assay and compared with day 0. (**c**) Cultured SEB-1 cells were labeled with MHC class I surface protein-specific antibodies, DAPI, and CD107a antibody, then imaged using confocal fluorescence microscopy. Scale bar = 50 μm. (**d**) Cultured SEB-1 cells were pretreated with NH_4Cl (1 mmol/L or 10mmol/L) or medium alone for 1 hour before stimulation with *P. acnes* (MOI = 10). Viable intracellular bacterial load was assessed using CFU assay after 24 hours, and relative percent viability was calculated based on initial inoculum. Data shown are the mean of CFU/cell of triplicate wells ± standard deviation, representative of three individual experiments. $^{**}P < 0.01$. Post hoc two-tailed Student *t* test was used for comparison between two groups. CFU, colony-formation unit; MHC, major histocompatibility complex; MOI, multiplicity of infection; NH_4Cl, ammonium chloride.

cultured SEB-1 cells indeed express lysosomes, shown by labeling with lysosomal-associated membrane protein 1 (LAMP1 or CD107a) (Figure 1c). We next assessed the functional role of these lysosomes by inhibiting the lysosomal pathway with ammonium chloride (NH_4Cl), which inhibits lysosome migration and phagosome-lysosome fusion (Hart et al., 1983). The addition of NH_4Cl, in a concentration-dependent manner, inhibited lysosomal function in SEB-1 cells and resulted in significantly more viable *P. acnes* after 24 hours (Figure 1d).

Innate cells also induce antimicrobial response via induction of endogenous AMPs. Human beta defensin-2 (hBD2) has been shown to be an important antimicrobial response against skin pathogens (Harder et al., 1997; Nakatsuji et al., 2010). We therefore investigated whether hBD2 induction was also involved in SEB-1 cells' antimicrobial response. We stimulated SEB-1 cells with *P. acnes* and measured the expression of the hBD2 gene, *DEFB4*. Because *P. acnes* has been shown to induce innate response via the TLR2-dependent pathway (Kim, 2005), we sought to determine whether the induction of AMPs in sebocytes was also TLR2-dependent. We found that *P. acnes* induced *DEFB4* (Figure 2a) and that the expression of *DEFB4* is dependent on TLR2, because induction of *DEFB4* by *P. acnes* was inhibited by 60% in the presence of neutralizing anti-TLR2 antibody.

To specifically evaluate antimicrobial activity related to TLR2, we stimulated SEB-1 cells with Pam3Cys, a TLR1/TRL2 agonist (TLR2/1L) before infection with *P. acnes*. Pam3Cys is commonly used to study TLR2 activity, although *P. acnes* itself activates TLR2 independently of TLR1. Treatment of sebocytes with TLR2/1L enhanced antimicrobial activity by 40% compared with medium alone (Figure 2b), indicating increased antimicrobial activity mediated through TLR2. Because *P. acnes* induced *DEFB4* in a TLR2-dependent manner, we evaluated its role in the enhanced antimicrobial activity induced by TLR2/1L. Transfection of SEB-1 cells with small interfering RNA oligonucleotides complementary to *DEFB4* mRNA eliminated the enhanced antimicrobial activity induced by TLR2/1L, with bacterial viability similar to medium control (Figure 2b and c). In cells transfected with the scrambled small interfering RNA control, antimicrobial activity was similar to that in nontransfected control cells, with 37% enhanced antimicrobial activity compared with medium control. Thus, these data suggest that *DEFB4* is involved in TLR2-mediated antimicrobial activity against *P. acnes*. Nonetheless, sebocytes transfected with *DEFB4* small interfering RNA maintained antimicrobial activity equal to media control, indicating that multiple alternative mechanisms also provide antimicrobial response in sebocytes.

Thus, we show that sebocytes exert an antimicrobial response against *P. acnes* in vitro through internalization and identify the lysosomal pathway and TLR2-mediated hBD2 induction as potential mechanisms of the antimicrobial activity observed. Our growing understanding of sebocytes portrays these skin-resident cells as more complicated

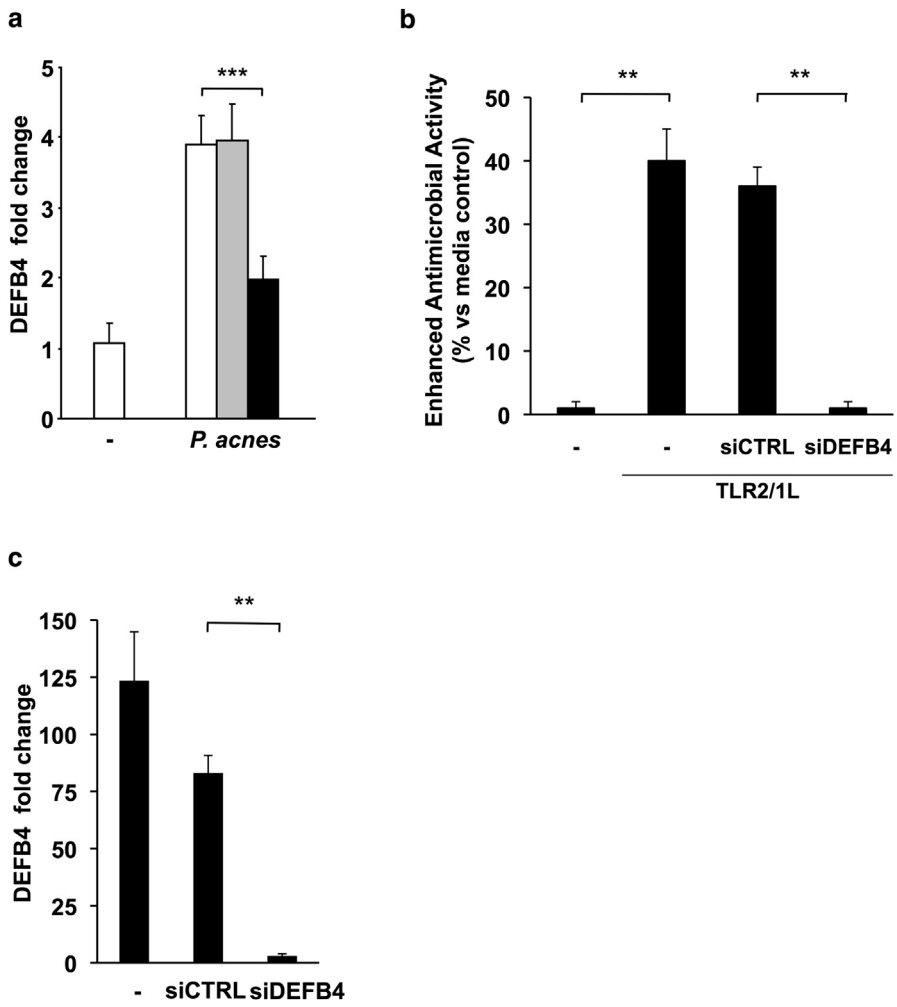

Figure 2. Antimicrobial activity against *P. acnes* based on antimicrobial peptides. (**a**) Cultured SEB-1 cells were pretreated with medium (open bar), 10μg/ml isotype control antibody (shaded bar), or 10μg/ml TLR2-blocking antibody (filled bar) for 1 hour before stimulation with *P. acnes* (MOI = 5) or media for 24 hours. *DEFB4* mRNA levels were measured by real-time PCR and normalized to housekeeping gene *GADPH*. (**b**) Cultured SEB-1 cells were transfected with siDEFB4 or siCTRL. Sebocytes transfected with siDEFB4 or siCTRL and untransfected sebocytes were treated with medium or TLR2/1L for 24 hours before infection with *P. acnes* (MOI = 0.5). Cells were washed after 3 hours, and their intracellular bacterial viability was determined by CFU assay after 24 hours. Relative antimicrobial activity was calculated as medium control minus TLR2/1L value divided by the media control value, multiplied by 100. (**c**) Cultured SEB-1 cells were transfected with siDEFB4 or siCTRL. Baseline *DEFB4* gene expression levels were measured by real-time PCR and normalized to the housekeeping gene *GADPH*. Data shown are the mean of triplicate wells ± standard deviation, representative of three individual experiments. $^{**}P < 0.01$, $^{***}P < 0.001$. Post hoc two-tailed Student *t* test was used for comparison between two groups. CFU, colony-formation unit; MOI, multiplicity of infection; siCTRL, scramble control; siDEFB4, small interfering RNA oligonucleotides complementary to *DEFB4* mRNA.

than previously thought. Sebocytes can contribute to acne pathogenesis through sebum production and secretion of inflammatory cytokines, and they also possess properties that provide a mechanism for reduction of bacteria by exerting significant antimicrobial activity against *P. acnes* through internalization and production of antimicrobial peptides.

The innate immune system must rapidly recognize microbial pathogens and trigger inflammation and antimicrobial responses to limit infection. However, the very same mechanisms intended to mitigate pathogens can cause tissue injury when the immune system reacts abnormally, leading to clinical manifestation of disease and associated morbidity. In the case of acne, the normal skin commensal microbe, *P. acnes*, induces a host response with resultant inflammation, leading to pathology.

As immunocompetent cells, sebocytes may contribute to the balance between limiting *P. acnes* infection through induction of antimicrobial responses and inducing inflammation, thus contributing to tissue injury through a dysregulated immune response. Further understanding of the immune defense mechanisms used by sebocytes against *P. acnes* and the inflammation propagated by sebocytes may lead to development of new therapeutic targets and therapeutics in acne and other dermatologic conditions in which sebocytes play an active role. Further studies are needed not only to assess the clinical relevance of our in vitro findings but also to further characterize the role of sebocytes by myriad other immune features.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Cooper GM. Lysosomes. In: Cooper GM, editor. The cell: a molecular approach (2nd ed.). Sunderland, MA: Sinauer Associates; 2000.

Harder J, Bartels J, Christophers E, Schroder J-M. A peptide antibiotic from human skin. Nature 1997;387:861.

Hart PD, Young MR, Jordan MM, Perkins WJ, Geisow MJ. Chemical inhibitors of phagosome-lysosome fusion in cultured macrophages also inhibit saltatory lysosomal movements. A combined microscopic and computer study. J Exp Med 1983;158:477–92.

Kim J. Review of the innate immune response in acne vulgaris: activation of Toll- like receptor 2

in acne triggers inflammatory cytokine responses. Dermatology 2005;211:193–8.

Kligman A. An overview of acne. J Invest Dermatol 1974;62:268–87.

Knutson D. Ultrastructural observations in acne vulgaris: the normal sebaceous follicle and acne lesions. J Invest Dermatol 1974;62:288–307.

Nagy I, Pivarcsi A, Kis K, Koreck A, Bodai L, McDowell A, et al. Propionibacterium acnes and lipopolysaccharide induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes. Microbes Infect 2006;8:2195–205.

Nagy I, Pivarcsi A, Koreck A, Szell M, Urban E, Kemeny L. Distinct strains of Propionibacterium acnes induce selective human beta-defensin-2 and interleukin-8 expression in human keratinocytes through toll-like receptors. J Invest Dermatol 2005;124:931–8.

Nakatsuji T, Chiang HI, Jiang SB, Nagarajan H, Zengler K, Gallo RL. The microbiome extends to subepidermal compartments of normal skin. Nat Commun 2013;4:1431.

Nakatsuji T, Kao MC, Zhang L, Zouboulis CC, Gallo RL, Huang CM. Sebum free fatty acids enhance the innate immune defense of human sebocytes by upregulating beta-defensin-2 expression. J Invest Dermatol 2010;130:985–94.

Molecular Profiling of Immune Activation Associated with Regression of Melanoma Metastases Induced by Diphencyprone

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TO THE EDITOR

Diphencyprone (DPCP), a hapten that causes delayed-type hypersensitivity reactions, has been used to treat cutaneous metastases in melanoma patients. An 84% regression rate was observed in a case series of 50 patients who had cutaneous melanoma metastases treated with multiple topical DPCP applications (Damian et al., 2014). However, the immunological mechanisms underlying these cases of regression are not well understood. Previously, our group characterized normal skin reactions in healthy volunteers upon a single application of topical DPCP gel (Gulati et al., 2014). Several immune pathways that we found to be induced by DPCP in normal skin, including IFN-γ and granulysin, may mediate the antimelanoma responses that have been observed clinically. In this study, we used the same topical DPCP formulation, which we previously applied only once to healthy volunteers, and applied it multiple times to patients with cutaneous melanoma metastases. Our cohort included six melanoma patients, of whom five exhibited partial or complete melanoma metastasis regression in response to DPCP treatment. Written informed consent was obtained from all subjects, and the study adhered to the Declaration of Helsinki Principles. By both immunohistochemical and gene expression approaches, we comprehensively characterized the immune reactions induced by DPCP in these patients. We present results from five of six patients treated with DPCP, excluding patient 001, who left the trial before a delayed-type hypersensitivity reaction could be successfully induced (see Supplementary Materials and Methods and Supplementary Table S1 online). All five patients showed partial or complete regression of their cutaneous metastases upon DPCP treatment (see Figure 1 for an example of complete clinical and MLANA immunohistochemical response). Further evidence of successful melanoma regression came from profiling of the global set of gene expression changes in these reactions by microarray analysis (data deposited in the NCBI's Gene Expression Omnibus, GSE accession number GSE82105). Because the five patients received different numbers of repeated applications of DPCP, we defined each patient's final biopsy sample as "chronic." When comparing the chronic biopsy samples with the pre-DPCP metastasis biopsy samples, many of the most significantly down-regulated genes were hallmark melanoma or melanocyte genes, such as *PRAME, TYR, OCA2, DCT,* and *MLANA,* which were down-regulated 12- to 22-fold (Table 1).

To better ascertain the mechanisms involved in immune-mediated tumor regression induced by DPCP, we studied biopsy tissue samples from the patients at various time points. In line with our healthy volunteer data (Gulati et al., 2014), DPCP applications in melanoma patients led to extensive immune cell infiltrates, including $CD3^+$ T cells, $CD11c^+$ myeloid dendritic cells, and $CD163^+$ macrophages, both after a single and repeated applications. These infiltrates persisted in follow-up biopsies performed 30 days after cessation of DPCP treatment (see Supplementary Figure S1a online). In addition to these cells, which are presumably integral to immune-mediated antimelanoma responses, we found that DPCP application led to increases in granulysin. By two-color immunofluorescence, granulysin co-localized with $NKp46^+$ natural killer cells more than $CD8^+$ cytotoxic T cells (see Supplementary Figure S1b). However, based on quantitative reverse transcriptase–PCR analysis, the levels of induction of various immune effectors, including IFNG and GNLY, after one application of DPCP were significantly lower in the melanoma patients than in the corresponding skin sites of healthy volunteers previously studied (Figure 1b), thus suggesting background immune suppression in the setting of melanoma, along with possible age-related changes because the melanoma patients tended to be older than the healthy volunteers.

Because the down-regulation of the five "melanoma signature" genes found

Abbreviations: DPCP, diphencyprone; PD-1, programmed cell death protein 1; Th, T helper

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