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Pseudomonas Aeruginosa Theft Biofilm Require Host Lipids of Cutaneous Wound

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Objective: This work addressing complexities in wound infection, seeks to test the reliance of bacterial pathogen *Pseudomonas aeruginosa* (PA) on host skin lipids to form biofilm with pathological consequences.

Background: PA biofilm causes wound chronicity. Both CDC as well as NIH recognizes biofilm infection as a threat leading to wound chronicity. Chronic wounds on lower extremities often lead to surgical limb amputation.

Methods: An established preclinical porcine chronic wound biofilm model, infected with PA or *Pseudomonas aeruginosa* ceramidase mutant ($PA_{\Delta Cer}$), was used.

Results: We observed that bacteria drew resource from host lipids to induce PA ceramidase expression by three orders of magnitude. PA utilized product of host ceramide catabolism to augment transcription of PA ceramidase. Biofilm formation was more robust in PA compared to $PA_{\Delta Cer}$. Downstream products of such metabolism such as sphingosine and sphingosine-1-phosphate were both directly implicated in the induction of ceramidase and inhibition of peroxisome proliferator-activated receptor (PPAR)δ, respectively. PA biofilm, in a ceram-idastin-sensitive manner, also silenced PPARδ *via* induction of miR-106b. Low PPARδ limited ABCA12 expression resulting in disruption of skin lipid homeostasis. Barrier function of the wound-site was thus compromised.

Conclusions: This work demonstrates that microbial pathogens must co-opt host skin lipids to unleash biofilm pathogenicity. Anti-biofilm strategies must not necessarily always target the microbe and targeting host lipids at risk of infection could be productive. This work may be viewed as a first step, laying fundamental mechanistic groundwork, toward a paradigm change in biofilm management.

Keywords: bacterial biofilm, cutaneous barrier function, host lipids, host-pathogen interaction, porcine wound model

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Bacterial biofilms complicate wound healing.[1–3] The Center for Disease Control estimates that 65% of all human infections are caused by bacteria with biofilm phenotype and the National Institutes of Health estimates that this number is closer to 80%.[4] Majority of chronic wounds are known to be infected by *Pseudomonas aeruginosa* (PA). Importantly, of all bacterial biofilm aggregates, those of PA are the largest.[5] PA infection is known to cause wound chronicity.[6] PA is equipped with repertoire of virulence determinants and a complex regulatory network of intracellular and intercellular signals[7] that allow the bacteria to adapt, thrive, and escape host defense.[8] They can live as free-living planktonic cells or as members of a biofilm community and have the exceptional ability to translate microbial signals and environmental cues into niche-specific processes.[9]

In the setting of host-microbe interaction, mechanistic underpinnings of biofilm infection are contextual and depend on the host tissue microenvironment. In this work, microbial biofilm involving larceny of host factors towards bolstering of underlying formative mechanisms, and worsening of host pathogenicity, is viewed as theft biofilm. Local tissue biochemistry as well as immune defense responses both influence such mechanisms.[10] Thus, translationally relevant understanding of wound biofilm infection may only be acquired from immune-competent preclinical models especially when delineation of time-dependent cascade of events is of interest.[1–3] Such approach enables the investigation of bacteria of clinical interest, such as PA AN17 strain.[11] Bacteria produce lipases which hydrolyze host esters of glycerol with fatty acids. In a skin wound microenvironment this is of outstanding significance. Epidermal lipids are a mixture of ceramides, free fatty acids, and cholesterol. Ceramides are a major lipid constituent, accounting for 40% to 50% of the cutaneous lipids by weig ht.[12] Exogenous fatty acids are known to contribute to PA pathogenicity by altering bacterial membrane phospholipid structure, membrane permeability, virulence pheno-types, and consequent stress responses that augment survival and persistence of these bacteria.[13] In this study, we sought to investigate the interaction between host skin lipids and bacterial factors capable of metabolizing them with the overall goal to understand how such interaction may determine biofilm formation. Findings of this work lend credence to a "theft biofilm" paradigm wherein massive induction of PA ceramidase by host skin lipids establish a loop whereby host factors are "stolen" to induce bacterial ceramidase transcription toward impaired functional wound closure.

METHODS

Detailed Methodology in Supplementary Information

Animals

All animal (pig) experiments were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (SoM-IACUC) and Ohio State University Institutional Laboratory Animal Care and Use Committee (ILACUC) under protocols 18048 and 2008A0012, respectively.

Bacterial strains

Pseudomonas aeruginosa wild type strain (PA_{wt}), *Pseudomonas aeruginosa* ceramidase mutant ($PA_{\Delta cer}$) were grown on Luria Agar (LA) plates or Luria broth with low sodium chloride (LBNS) at 37°C.[1,2]

Porcine Full Thickness Burn and Biofilm Wound Model

Domestic Yorkshire female pigs were wounded and infected to establish chronic wound biofilm model as described previously.[1–3,14] Eight full thickness burn wounds were made and infected with culture comprising of PA_{wt},or $PA_{\Delta cer}$ strains (CFU10^5/mL) with *Acinetobacter baumannii* (AB) (CFU 10^6/mL) in both groups or allowed to be colonized by skin microflora and referred as spontaneously infected (SI).[2] Wounds were followed up to 56 days postinfection. Details in Supplemental Information.

Scanning Electron Microscope Imaging

Sample processing and imaging was performed as described previously.[1] Details in Supplemental Information.

Transepidermal Water Loss Measurement

DermaLab Combo (cyberDERM inc., Broomall, PA) was used to measure the trans-epidermal water loss from the wounds.[2] Trans-epiderma water loss (TEWL) was measured in g/m$^{2/}$h.

Bacterial Ceramidase activity

Pseudomonas ceramidase activity assay was adapted from Ohnishi *et al.*[15] Details in Supplemental Information.

Lipidomic Analyses Using LC ESI-MS/MS

Targeted and untargeted analysis of the sphingolipidome was undertaken as previously described.[16–18] Details in Supplemental Information.

Lipidomic Analysis of Sphingosine-1-Phosphate

Porcine wound edge tissue pulverized samples were spiked with 20 µL of ceramide/sphingolipid mixture I (Avanti) with 0.5 nmol of d17:1–P. The lipids were extracted using a modified Bligh and Dyer method.[17] Quantitation was based on Multiple Reaction Monitoring (MRM). Details in Supplemental Information.

In Vitro Pseudomonas Biofilm Model

In vitro biofilm culture on polycarbonate membrane was adapted from Zhao et al, 2010.[19] *In vitro* biofilm was grown on a polycarbonate membrane (PCM) by inoculating PA_{wt}, or $PA_{\Delta cer}$ bacteria cells (CFU 10^5/mL) with or without porcine skin lipids for 24 hours. Details in Supplemental Information.

Immunohistochemistry

Immunohistochemical staining of the frozen sections and immunocytochemistry were performed using standard procedures[2] Antibodies listed in Table S3, http://links.lww.com/SLA/D496.

Nile Red Staining

The OCT embedded wound tissue sections were stained with nile red as described previously.[20] Details in Supplemental Information.

Wheat-Germ Agglutinin Staining

The PCM disc with *in vitro* bacteria biofilm were WGA -Alexa Fluor 488 Conjugate as described previously.[21] Details in Supplemental Information.

Western Blot Analyses

Western blot was performed using antibodies against anti-peroxisome proliferator-activated receptor (PPAR)B and anti-CerS3. ß-actin was used as housekeeping. Antibodies listed in Table S3, http://links.lww.com/SLA/D496.

PPARδ Trans-Activity Assay

Human keratinocytes cells were treated with 5 µmoL of long chain ceramides, C18:0 and C 24:0 (Avanti) and collected 48 hours post-treatment. Nuclear protein was extracted using Nuclear Extraction Kit (RayBiotech) and PPARB trans-activity was measured using PPAR delta Transcription Factor Assay Kit (Abcam) as per manufacturer's instructions. Details in Supplemental Information.

DNMT3B Activity Assay

Human keratinocytes cells were treated with 5 µmol Sphin-gosine-1-Phosphate (S–1–P) (Avanti) for 48 hours. Cells were collected and DNMT3B activity was measured using Epi-Quik DNMT3B Activity/Inhibitor Screening Assay Core Kit (Epigentek) according to manufacturer's instructions and using recombinant DNMT3B protein, (Active motif) as a positive control. Details in Supplemental Information.

PPARδ Promoter Assay

PPARδ promoter assay was done as follows. mNP-Luc (PPARdelta promoter) (Addgene) reporter construct was used. Human keratinocytes cells were co-transfected with the PPARB promoter reporter construct and 5 µmol of C:18 and C:24. Luciferase assay and normalization was performed using the dual-luciferase reporter assay system (Promega). Data are presented as the ratio of firefly: renilla.[2]

miR-Target 3′-UTR Reporter Assay

Human keratinocytes were transfected with miRIDIAN mimic-miR–106b followed by transfection with miR target

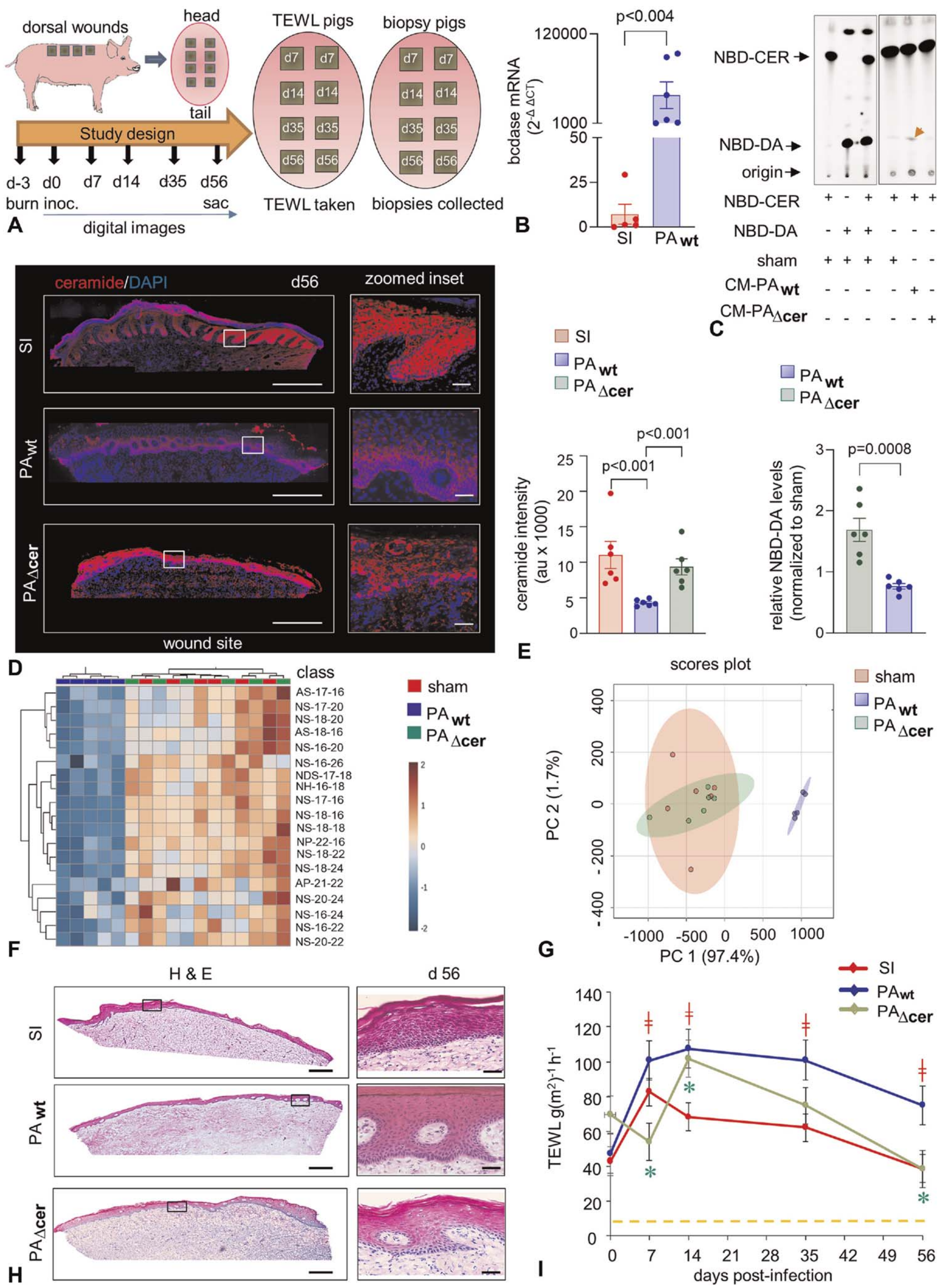

FIGURE 1. Inducible Bacterial Ceramidases Deplete Host Cutaneous Ceramide. (A) Schematic presentation of the timeline of porcine model of chronic wound biofilm infection. (B) Induction of bacterial ceramidase *(bcdase)* in biofilm infected porcine wounds. Real-time qPCR analysis of *bcdase* expression spontaneously infected (SI) and wild type *P. aeruginosa* (PA_{wt}) infected

PPARδ-3'–UTR plasmid (NM_006238) or CerS3–3'–UTR plasmid (NM_178842). Luciferase assay and quantification done as mentioned above. Details in Supplemental Information.

miRNA Delivery

Transfection of human keratinocytes cells was performed as described.[2] Details in Supplemental Information.

Bisulfite Conversion of DNA Sequencing of PPARδ Promoter

Bisulfite conversion of DNA and sequencing of S-1-P trans-fected human keratinocytes was performed as described previously.[22] Primers for sequencing PPARδ promoter region listed in Table S2, http://links.lww.com/SLA/D496. Details in Supplemental Information.

Quantification and Statistical Analysis

The data analysis was performed using student *t* test (2-tailed) presented as mean ± SEM. Mean, SEM, and student *t* test analyses were done using in-built function in Microsoft Excel 2010.

Comparisons among multiple groups were tested using ANOVA in built function in GraphPad Prism 9.1.2. $P < 0.05$ was considered statistically significant.

RESULTS

Wound Biofilm Infection Depletes Host Skin Ceramides

In an established pre-clinical porcine chronic wound biofilm model[2] (Fig. 1A, Table S1, http://links.lww.com/SLA/D496), the expression of PA ceramidase was induced by 3 orders of magnitude on day 7 following infection with PA_{wt} (Fig. 1B). Control wounds were not subjected to induced infection and were allowed to be colonized by natural skin microflora. These wounds are referred to as spontaneous infection (SI).[2] Bacterial ceramidase is known to cause breakdown of host ceramides.[15] To determine the significance of PA ceramidase, a ceramidase-deficient $PA_{\Delta Cer}$ was studied. The loss of ceramidase *(bcdase)* gene in the mutant bacterial strain was validated using qRT PCR (Fig. S1A, http://links.lww.com/SLA/D496). Loss of ceramidase activity was evident in $PA_{\Delta Cer}$ as measured by thin layer chromatography (TLC) using a fluorescent ceramide analog (Fig. 1C). PA and AB infection was confirmed by CFU assay on *Pseudomonas aeruginosa* selection agar and *Acinetobacter* selection agar (Figs. S1B, http://links.lww.com/SLA/D496, S2, http://links.lww. com/SLA/D496).

To identify bacterial species and their abundance, a 16S rRNA (variable region) next generation sequencing (NGS) was performed. PA infection was further confirmed on the basis of NGS sequencing (Fig. S1C, http://links.lww.com/SLA/D496). Although the initial infection was polymicrobial (PA + AB), over time PA prevailed as dominant biofilm species (Figs. S1C, http://links.lww.com/SLA/ D496, S2E-F, http://links.lww.com/SLA/D496) as determined by NGS and CFU assays (Fig. S1C, http://links.lww.com/SLA/D496, S2, http://links.lww.com/SLA/D496). To test whether the reported effects are causatively linked to AB infection, we used group of pigs that was infected with PA_{wt} alone, and another group was infected with AB alone. Ceramide abundance was measured in the wound tissue d56 post-infection. Ceramide depletion was limited to wounds infected with PA and was not evident in response to AB infection (Fig. S3A-B, http://links.lww.com/SLA/D496). These data are consistent with our published IHC/MALDI-TOF studies characterizing pig wound infection.[1, 3] The formation of bacterial biofilm aggregates was validated using scanning electron microscopy (SEM) and staining with PA biofilm matrix component Pel-specific *Wisteria floribunda* lectin (WFL) staining[23] (Fig. S1D, http://links.lww.com/SLA/ D496, S1E-F, http://links.lww.com/SLA/D496).

Biofilm-dependent loss of skin ceramide was evident in PA_{wt}, but not in $PA_{\Delta Cer}$ (Fig. 1D and E). In vitro studies identified that exposure to host skin lipids potently induced *bcdase* in PA_{wt}; such induction was attenuated in PA_{wt} exposed to depleted skin lipids (Fig. S1G, http://links.lww.com/SLA/D496). Inducible *bcdase* was associated with the induction of the *Pseudomonas* SphR gene (Fig. S1H-I, http://links.lww.com/SLA/D496). SphR is known to function as the transcriptional activator of *bcdase.*[24]

Biofilm-dependent loss of skin ceramides was characterized employing a lipidomics approach. Nineteen long-chain cutaneous ceramides were observed to be depleted in response to biofilm infection by PA_{wt}, but not $PA_{\Delta Cer}$ (Fig. 1F, Fig. S4A-S, http://link-s.lww.com/SLA/D496). Principal component analyses (PCA) revealed that the abundance of cutaneous ceramides in response to PA_{wt} was statistically distinct from the cluster of cutaneous ceramide levels in response to $PA_{\Delta Cer}$ and sham exposure (Fig. 1G). Mechanistic studies addressing the loss of keratinocyte ceramide following PA_{wt} biofilm infection were conducted in vitro (Fig. S4T, http://links.lww.com/SLA/D496). Ceramidastin, an inhibitor of PA ceramidase,[25] rescued keratinocyte ceramide against loss caused by PA_{wt} biofilm infection

porcine wound epidermis (d7 post-infection) collected by laser capture micro-dissection (LCM). Data presented as mean ± SEM, (n = 5). (C) $PA_{\Delta cer}$ biofilm was deficient in secreted *bcdase* activity. Data presented as mean ± SEM, (n = 6). See Fig. S1A, http:// links.lww.com/SLA/D496 for gene *bcdase* expression data. [NBD-CER - C12-NBD-Ceramide, NBD-DA - NBD-dodecanoic acid]. (D and E) Depletion of ceramide in porcine wounds infected with PA_{wt} biofilm compared to SI or wounds infected with ceramidase mutant of *P. aeruginosa* ($PA_{\Delta cer}$). Porcine d56 wound tissues were immuno-stained with anti-ceramide (red) antibody and DAPI (blue). Data presented as mean ± SEM, (n = 6). Scale bar = 500 μm and zoomed inset = 50 μm. (F) Depletion of ceramides in porcine skin lipid exposed to PA_{wt},or $PA_{\Delta cer}$ as measured by LC/MS/MS targeted-lipidomic approach. Same skin lipid not Exposed to any bacteria was used as sham control. Hierarchical cluster analysis revealed down-regulation of 19 species of ceramide in the skin lipid (N = 6). Individual ceramides that were specifically affected are illustrated in Supplementary Fig. S4, http:// links.lww. com/sla/d496. (G) Principal component analyses of the abundance of cutaneous ceramides in response to PA_{WT} or $PA_{\Delta CER}$ exposure. Sham and $PA_{\Delta CER}$ clusters were not statistically different. This combined group was statistically distinct from cutaneous ceramide levels in response to PA_{WT}. (H) Complete re-epithelization following SI, PA_{WT} and $PA_{\Delta CER}$ biofilm infection. Hematoxylin-eosin staining shown. Scale bar = 500 μm and zoomed inset = 50 μm. Macroscopic digital planimetry shown in Supplementary Fig. S5M, http://links.lww.com/sla/d496. (I) functional restoration of Barrier function of cutaneous wounds, measured by tewl, was compromised following PAWT biofilm infection. Data presented as mean ± SEM (N = 6). / $P < 0.05$ as compared to $PA_{\Delta CER}$; and *$P < 0.05$ as compared to SI.

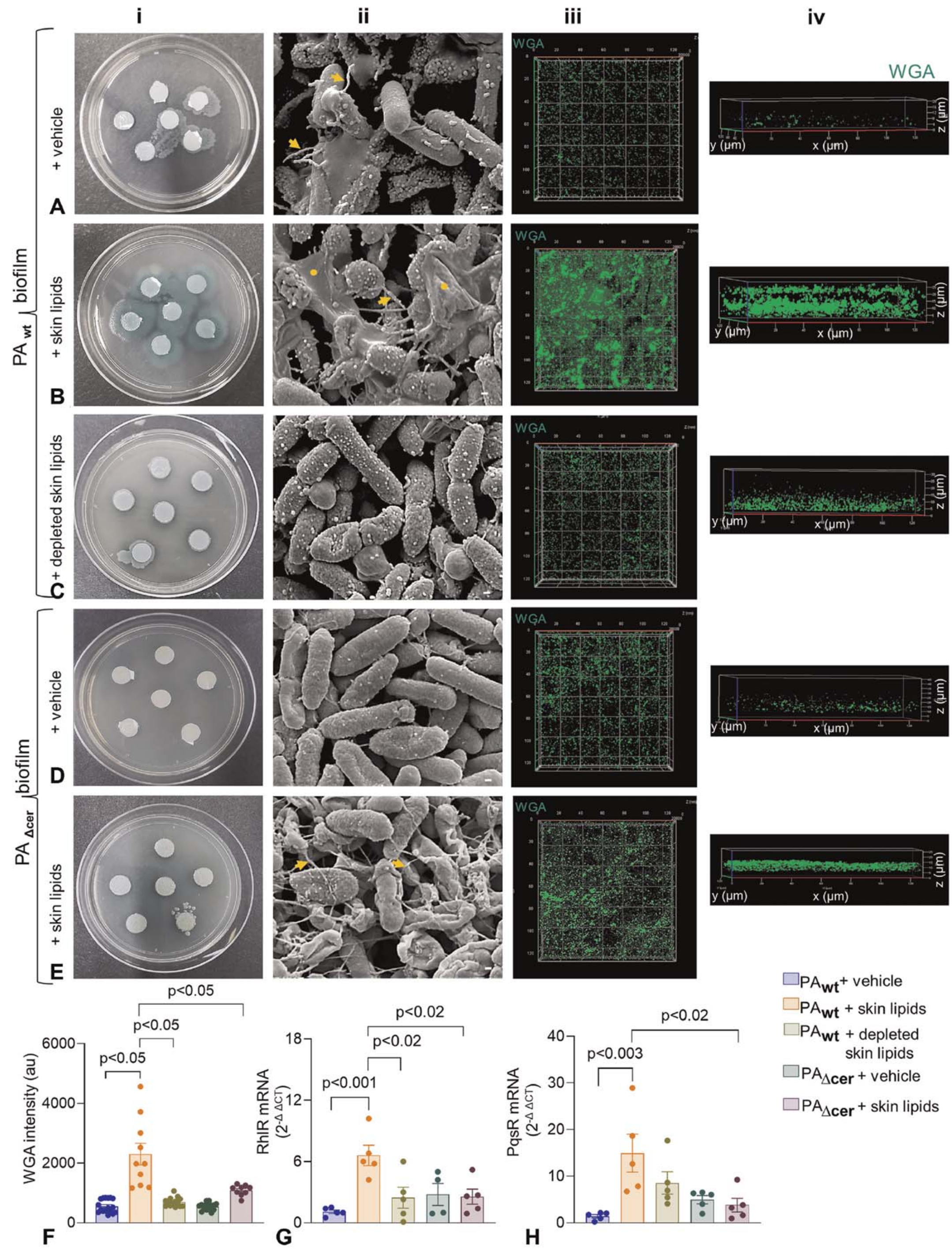

FIGURE 2. Host Lipids Facilitate PA_{wt} Biofilm Aggregate Formation. (A-E) (i) Cutaneous lipids induced biofilm aggregate formation in an *in vitro* polycarbonate membrane biofilm system after 24 h of inoculation. A, PA_{wt} +vehicle (PBS); B, PA_{wt} + skin lipids; C, PA_{wt} + depleted lipids; D, $PA_{\Delta cer}$ +vehicle (PBS) E, $PA_{\Delta cer}$ + skin lipids. (ii) Increased abundance of EPS in PA_{wt} biofilm in response to host lipids as recorded in SEM images. Thick EPS is marked by yellow circles. EPS fibers are marked by yellow arrows. Scale bar = 100 nm, 30,000x magnification. Larger SEM fields are presented as supplementary Figure 6, http://links.lww.com/SLA/D496. (iii) Host lipids induced biofilm aggregate formation in PA_{wt} as measured by wheat germ agglutinin (WGA) staining. Scale bar = 10 μm. (iv) Respective 3D reconstructed images of (iii). (F) Quantification of biofilm aggregates using WGA staining as shown in (iii). Data presented as mean ± SEM, n= 10. (G-H) Host lipids induce biofilm gene expression in pathogen PA_{wt}. Data presented as mean ± SEM, (n = 5).

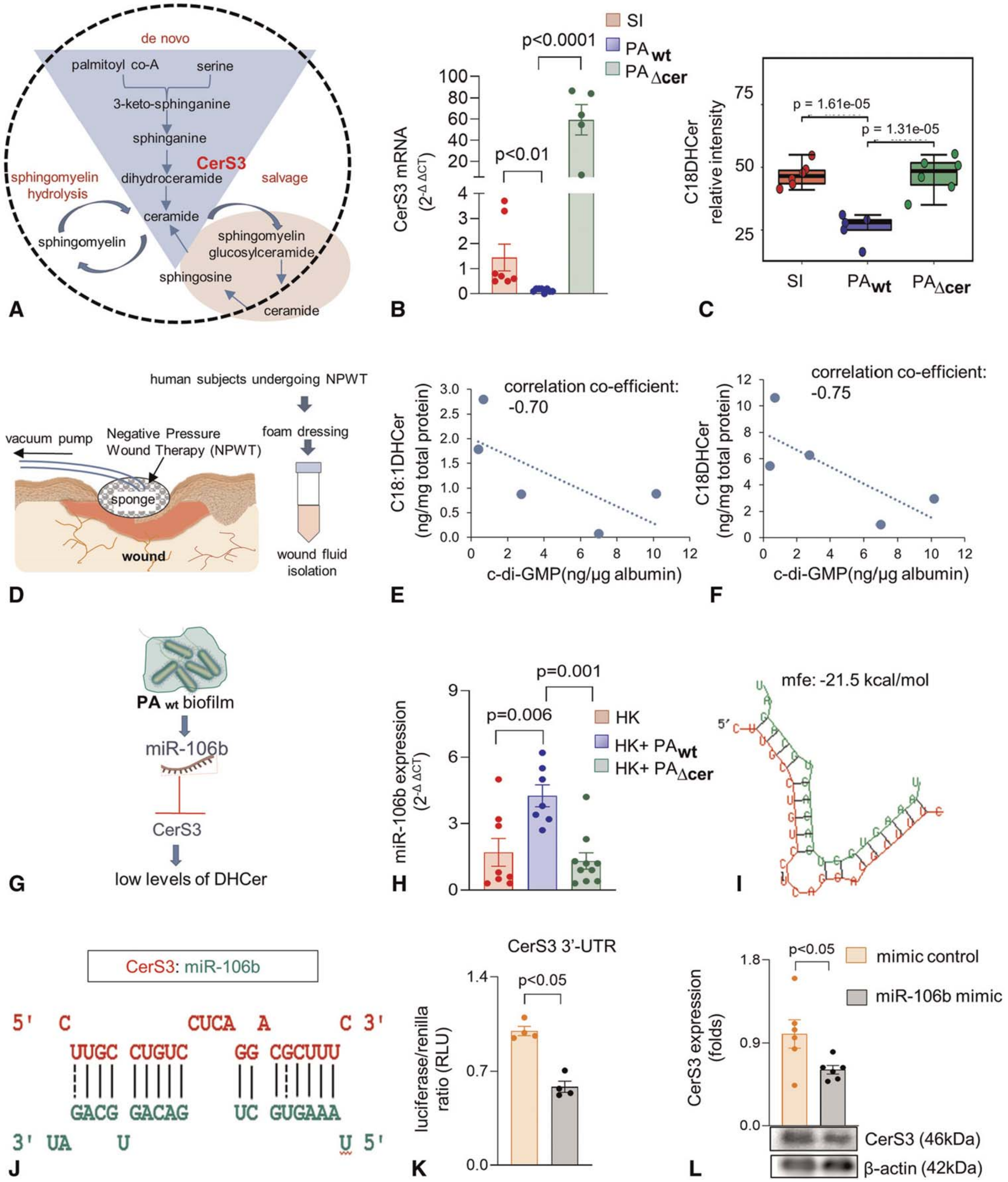

FIGURE 3. Biogenesis of host long chain ceramides is compromised under conditions of PA_{wt} Biofilm infection. (A) Long chain ceramide biogenesis pathways outlined. (B) Downregulation of CerS3 in wound-site skin tissue infected with PA_{wt} compared to SI or $PA_{\Delta cer}$ measured by qPCR. Data presented as mean ± SEM (n = 5-7). (C) PA_{wt} lowered the levels of dihydroceramide (C18DHCer) in skin lipids. Measured by LC/MS/MS targeted lipidomic approach. Data are presented as mean ± SEM (n = 6). (D) Wound fluid collection from chronic wound patients who underwent negative pressure wound therapy (NPWT) as part of standard of care. (E and F) Lower levels of dihydroceramide in cyclic diGMP-rich human wound fluid. cyclic di-GMP is a marker of PA biofilm infection (n = 5). (G) Biofilm induced miR-106b targets host CerS3 (H) Elevated miR-106b in human keratinocytes (HK) infected with PA_{wt} biofilm as measured by qPCR. Data presented as mean ± SEM (n = 7–10). (I and J) miR-106b is predicted to target the 3′–UTR of CerS3 position 331–355 according to RNAHybrid algorithm. (K) miR-106b silenced pmiR-Target-Cers–3′–UTR in human keratinocytes (HK). FL indicates Firefly luciferase;RL, Renilla luciferase. Data are mean ± SEM (n = 4). (L) miR-106b silenced CerS3 protein expression in human keratinocytes (HK). Data are presented as mean ± SEM (n = 6).

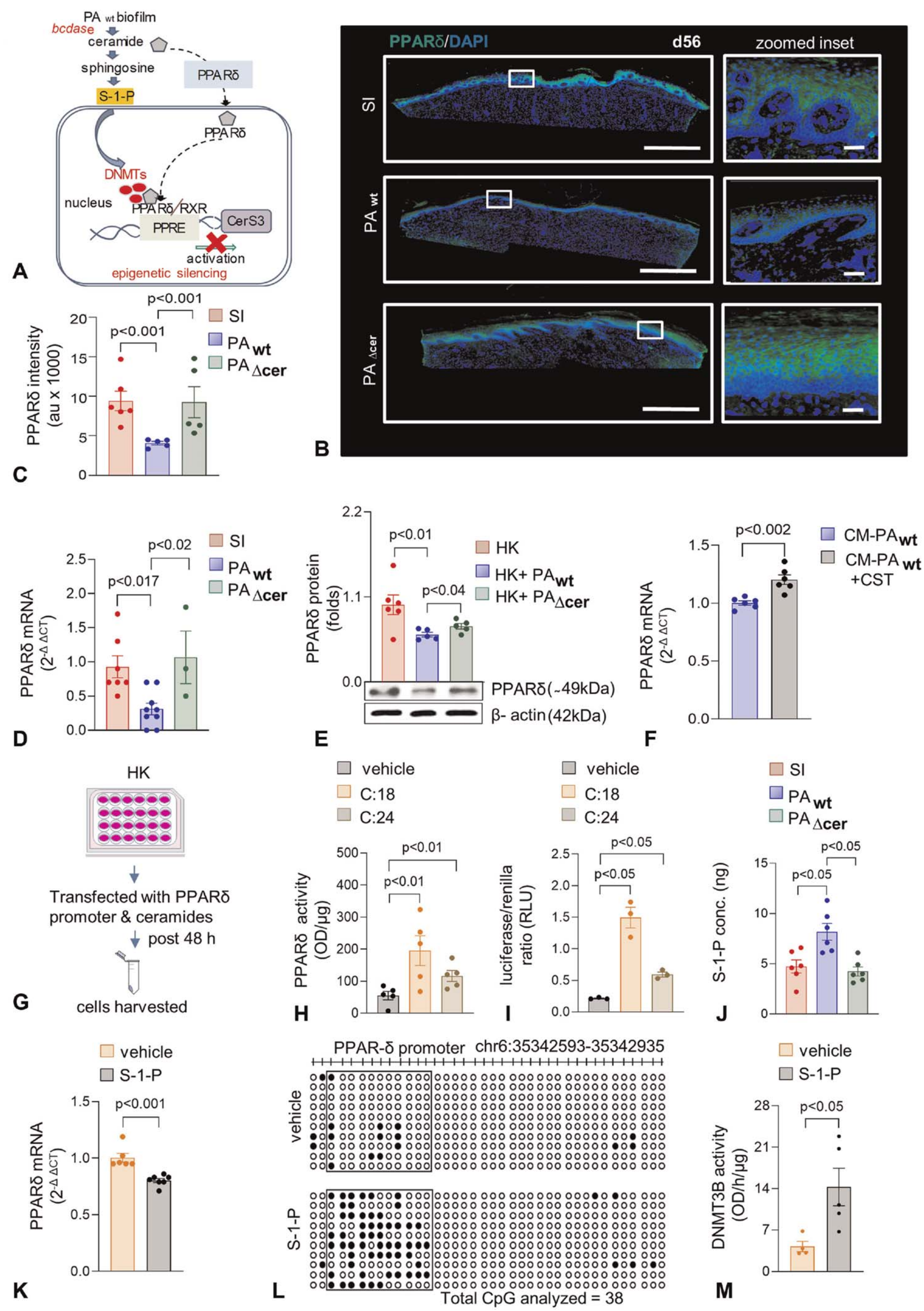

FIGURE 4. Wound-Site Skin PPARδ Expression is Downregulated in Response to PA^$_t$ Biofilm Infection. (A) PA_{wt} biofilm compromises host cutaneous PPARδ expression. (B and C) Loss of cutaneous host PPARB in porcine wounds infected with PAwt

(Fig. S4U, http://links.lww.com/ SLA/D496). Consistent data on rescue of ceramides by ceramidastin were observed in *ex vivo* studies on extracted skin lipids treated with biofilm-conditioned media (Fig. S5A-L, http://links.lww.com/SLA/ D496).

Wounds in all 3 groups of infection (SI, PA_{wt}, and $PA_{\Delta Cer}$) were studied over a period of 56 days. During this period, all of these wounds were completely closed as evident by wound planimetry (Fig. S5M, http://links.lww.com/SLA/D496) as well as histology (Fig. 1H, Fig. S3C, http://links.lww.com/SLA/ D496). In such experimental setting, we sought to determine the functional significance of biofilm-dependent depletion of skin ceramides. Skin barrier integrity was studied by measuring TEWL. Under conditions of PA_{wt} biofilm infection, depletion of skin ceramides was associated with elevated TEWL (Fig. 1I). Such impairment in skin barrier function was not observed in response to $PA_{\Delta Cer}$ (Fig. These findings establish a causal relationship between induction of *bcdase* and inability of the repaired skin to restore barrier integrity.

Skin Lipids Augment Biofilm Via Bcdase

Study of skin tissue extract, native or lipid-depleted (Fig. S6A-B, http://links.lww.com/SLA/D496), demonstrated clear role of lip-ids in augmenting biofilm formation. To test the significance of breakdown of skin ceramides on biofilm formation, extracted lipids were added to bacteria that were sufficient or deficient in *bcdase.* Addition of skin lipid extract markedly enhanced biofilm formation in PA_{wt} as measured by SEM and EPS staining (Fig. 2A–C). Such augmentation was blunted in $PA_{\Delta Cer}$ (Fig. 2D and E). This pointed toward a likely role of skin ceramide degradation products in biofilm formation (Fig. 2F, Fig. S6C-G, http://links.lww.com/SLA/D496). During biofilm formation, bacteria use quorum sensing (QS) to coordinate behaviors such as antibiotic resistance.[26] In PA, QS is driven by a series of small molecule receptors, including the master QS systems mvfR which is also known as pqsR and rhlR.[27] In line with structural observations on biofilm aggregates, the expression of pqsR, and rhlR, was markedly high in PA_{wt} biofilm treated with host lipids as compared to $PA_{\Delta Cer}$ under the same treatment conditions (Fig. 2G and H). An increased growth of $PA_{\Delta Cer}$ was observed (Fig. S6H, http://links.lww.com/SLA/ D496). This is in consistent with the in vivo observation where $PA_{\Delta Cer}$ exhibited faster growth. Although, PA_{wt} was a comparative slow grower in presence of host, it exhibited increased biofilm formation as documented through blue phenazine (pyocyanin) formation, WGA staining and crystal violet staining (Fig. 2B, Fig. S6I, http://links.lww.com/SLA/D496) and increased expression of *pqsR* gene (Fig. 2H). PqsR/mvfR is known to be required for pyocyanin formation.[28] Pyocyanin contributes to biofilm formation by facilitating extracellular DNA binding to PA.[29] These data thus demonstrated that the microbial pathogens are not capable of mounting the complex threat in isolation and that they must co-opt host skin lipids to generate complex biofilms.

Wound Biofilm Downregulates Host Skin CerS3 and Depletes Dihydroceramide

Skin ceramide homeostasis relies on a CerS3-dependent bio-synthetic pathway that produces long-chain ceramides[30] (Fig. 3A). Wound biofilm infection compromised ceramide biosynthesis by downregulating CerS3 expression in the PA_{wt} infected wounds, but not in $PA_{\Delta Cer}$ (Fig. 3B, S7A, http://links. lww.com/SLA/D496). Blunted cutaneous CerS3 expression was associated with depletion of long-chain dihydroceramide levels in the porcine skin tissue exposed to PA_{wt} infection (Fig. 3C). To determine the presence of PA biofilm, cyclic di-GMP was used as a surrogate marker in the wound fluids from chronic wound patients. Consistent with our findings from preclinical porcine studies, the investigation of wound fluid from chronic wound patients revealed tight correlation between lowering of long-chain dihydroceramide levels with elevated levels of a PA biofilm marker cyclic di-GMP (Fig. 3D-F, Fig. S7B-H, http:// links.lww.com/SLA/D496). Thus, PA biofilms, as marked by diGMP, were associated with lower levels of dihydroceramide.

Our previous work identified miR–106b as biofilm-inducible in wound-edge skin tissue with a pathogenic role.[2] In this work, miR-106b was induced in response to biofilm infection caused by PA_{wt}, but not by $PA_{\Delta Cer}$ (Fig. 3G and H). CerS3 is subject to post-transcriptional gene silencing by miRNA. RNAHybrid analyses revealed that the 3'–UTR of CerS3 is likely to be targeted by miR-106b (Fig. 3I and J). Biological validation of such prediction was conducted in human keratinocytes. Delivery of miR-106b mimic significantly lowered CerS3 3'–UTR reporter activity (Fig. 3K, Fig. S7I, http://links. lww.com/SLA/D496). Consistent with this finding, miR–106b mimic decreased CerS3 protein expression (Fig. 3L).

compared to SI or $PA_{\Delta cer}$ wounds. Porcine wound sections were immunostained with anti-PPARδ (green) and DAPI (blue). Porcine wound section images (scale bar = 500 μm) and corresponding zoomed inset (scale bar = 50 μm) showed PPARδ protein expression. Data presented as mean ± SEM (n = 5–6). (D) Lower expression of PPARδ in PA_{wt} infected wound tissue. Whole tissue homogenate was used for analysis. Data presented as mean ± SEM, (n = 3–8). (E) Lower expression of PPARδ protein in human keratinocytes (HK) infected with PA_{wt} biofilm. Data presented as mean ± SEM (n = 5). (F) Ceramidastin attenuated the negative effects of PA_{wt} biofilm on PPARB expression in human keratinocytes. Ceramidastin, 10 μg/mL. Data presented as mean ± SEM (n = 6). (G) PPARB promoter assay approach. (H) Increased PPARδ transactivity in human keratinocytes (HK) treated with long chain ceramides (C18 & C24, 5 μmol/mL). Nuclear extract of the transfected cells treated for 48 h were used to measure PPARB transactivity. Data presented as mean ± SEM (n = 5). (I) Activation of PPARδ promoter by long chain ceramides as measured by reporter assay. Human keratinocytes (HK) were transfected with PPARB promoter along with long chain ceramides (C:18, C:24, 5 μmol/ mL). Data presented as mean ± SEM (n = 3). (J) Elevated levels of sphingosine-1-phosphate (S-1-P) in PA_{wt} infected wound tissues. Measured by LC/MS/MS targeted lipidomic approach. Cutaneous wound tissue infected with PA_{wt} showed higher levels of S-1-P as compared to SI or $PA_{\Delta cer}$ infected wounds. Data presented as mean ± SEM (n = 6). (K) Down-regulation of PPARδ gene expression in human keratinocytes (HK) receiving S-1-P (5 μmol/mL). Data presented as mean ± SEM (n = 6-7). (L) Region of PPARδ promoter analyzed through bisulfite genomic sequencing of DNA. Methylation profile of the PPARB promoter in human keratinocytes (HK) treated with S-1-P (5 μmol, 48 h). Methylated CpG in black (filled) and unmethylated CpG in white (open). Number of clones = 10. (M) Increased DNA methyl transferase 3B (DNMT3B) activity in human keratinocytes (HK) treated S-1-P (5 μmol/L, 48h). Data presented as mean ± SEM (n = 4-5).

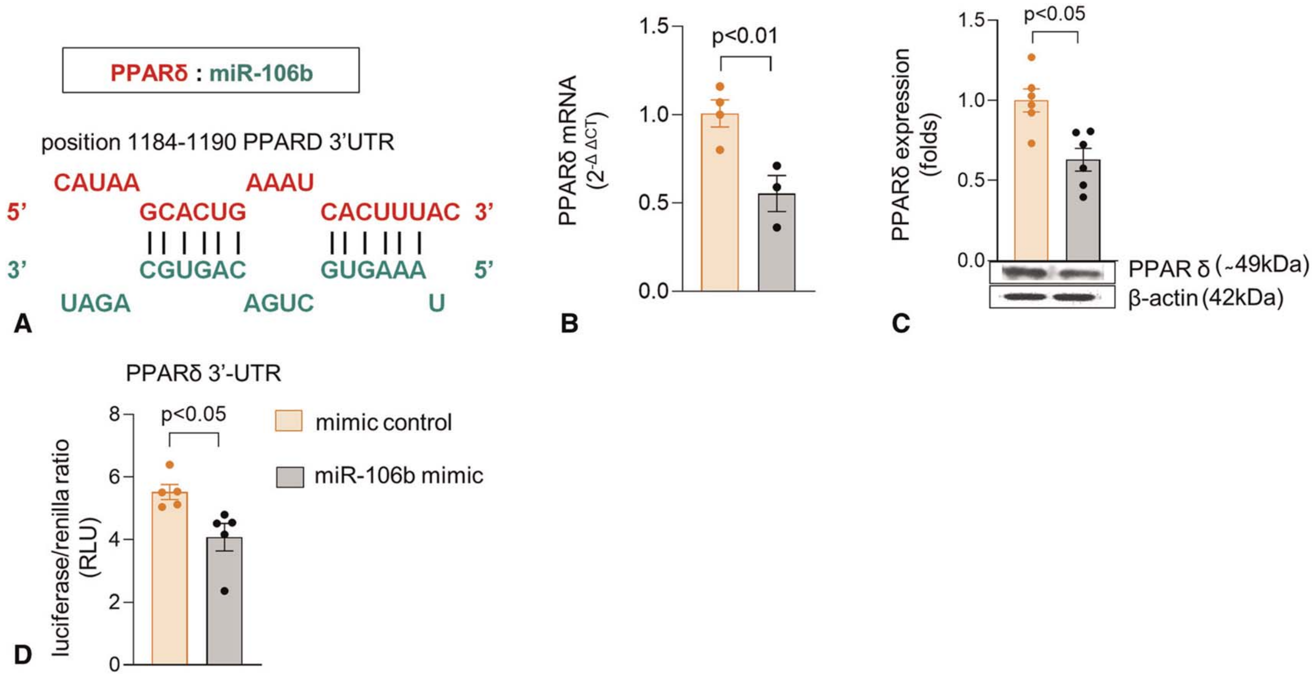

FIGURE 5. miR-106b Targets PPARB in PA_{wt} Biofilm Infected Keratinocytes. (A) RNA HybridTM-based prediction shows that PPARB 3'–UTR isa target of miR-106b. (B) PPARB downregulation in miR-106b mimic transfected human keratinocytes (HK) as measured by qPCR. Data presented as mean ± SEM (n = 3-4). (C) Downregulated PPARB protein in response to miR-106b mimic as measured by Western blot in HK. Data presented as mean ± SEM (n = 6). (D) miR-106B targets PPARB 3'UTR as shown by reporter assay. HK were transfected with HMIT013627 -MT06 - PPARΔ -3'-UTR (NM_006238) along with miR-106B mimic or control mimic. Data presented as mean ± SEM (N = 5).

Arrest of PPARΔ Activity Following Biofilm-Dependent Ceramide Depletion

In the peroxisome proliferator activated receptor (PPAR) family of transcription factors, PPARδ specifically is ceramide-sensitive.[31] In biofilm-affected ceramide-depleted tissue, PPARδ expression was downregulated. Such effect was not observed under conditions of $PA_{\Delta Cer}$ infection pointing towards a causative role of skin ceramide depletion (Fig. 4A–D). To address the underlying mechanisms, studies on human keratinocyte biofilm infection were conducted. Consistent with findings from porcine studies, biofilm infection blunted PPARd expression (Fig. 4E). Such effect was rescued in the presence of the PA ceramidase inhibitor, ceramidastin (Fig. 4F). Skin ceramides are primarily long-chain (≥C18). Thus, C18 and C24 ceramides were tested for their ability to regulate PPARδ function. In human keratinocytes, these long-chain ceramides induced PPARδ transactivation (Fig. 4G and H) as well as transcription (Fig. 4I, Fig. S8A, http:// links.lww.com/SLA/D496). PPARB agonist GW501516 increased expression of CerS3 (Fig. S8B, http://links.lww.com/SLA/D496), whereas its antagonist GSK0660 blunted the expression of CerS3 (Fig. S8C, http://links.lww.com/SLA/D496) in keratinocytes. These findings constitute evidence demonstrating direct regulation of PPARδ by ceramides in keratinocytes.

S-1-P Methylates PPARΔ Promoter

Cutaneous ceramide is degraded to sphingosine by bacterial ceramidase.[15] Sphingosine is phosphorylated to S-1-P, which is a bioactive lipid and can epigenetically downregulate gene regula-tion.[32] Thus, a plausible role of S-1-P in regulating PPARB in our experimental systems was tested. In the porcine preclinical model of wound biofilm infection elevated levels of S-1-P was detected following PA_{wt}, but not $PA_{\Delta Cer}$, infection (Fig. 4J). This finding indicated that the detected S-1-P was a breakdown product of skin ceramide acted upon by *bcdase.* Under standard culture conditions when human keratinocytes were treated with the bioactive sphingo-lipid S-1-P, gene expression of PPARB was blunted (Fig. 4K). To determine whether such downregulation of PPARB was epigeneti-cally regulated, CpG methylation of the PPARB promoter was studied. S-1-P caused promoter methylation (Fig. 4L). Such increased PPARδ promoter methylation was associated with augmented catalytic activity of DNA methyl transferase 3B (DNMT3B; Fig. 4M). A survey of the effects S-1-P on epigenetic regulators unveiled broader effects on gene expression favoring DNA methyl-ation and histone deacetylation (Fig. S8D-N, http://links.lww.com/ SLA/D496). Induction of miR-106b by S-1-P constitutes an additional epigenetic mechanism by which S-1-P may attenuate PPARδ (Fig. S8O, http://links.lww.com/SLA/D496).

PPARΔ is a Novel Target of Biofilm-Induced miR-106b

The search for PPARδ-targeting miRs, that were also biofilm-inducible, led to the identification of miR-106b as a candidate (Fig. 5A). Delivery of miR-106b mimic compromised PPARδ levels both at mRNA (Fig. 5B) and protein levels (Fig. 5C). PPARδ was further validated as a target for miR-106b by 3'UTR luciferase reporter assay (Fig. 5D). Systematic studies thus established PPARδ as a target of biofilm inducible miR–106b in keratinocytes.

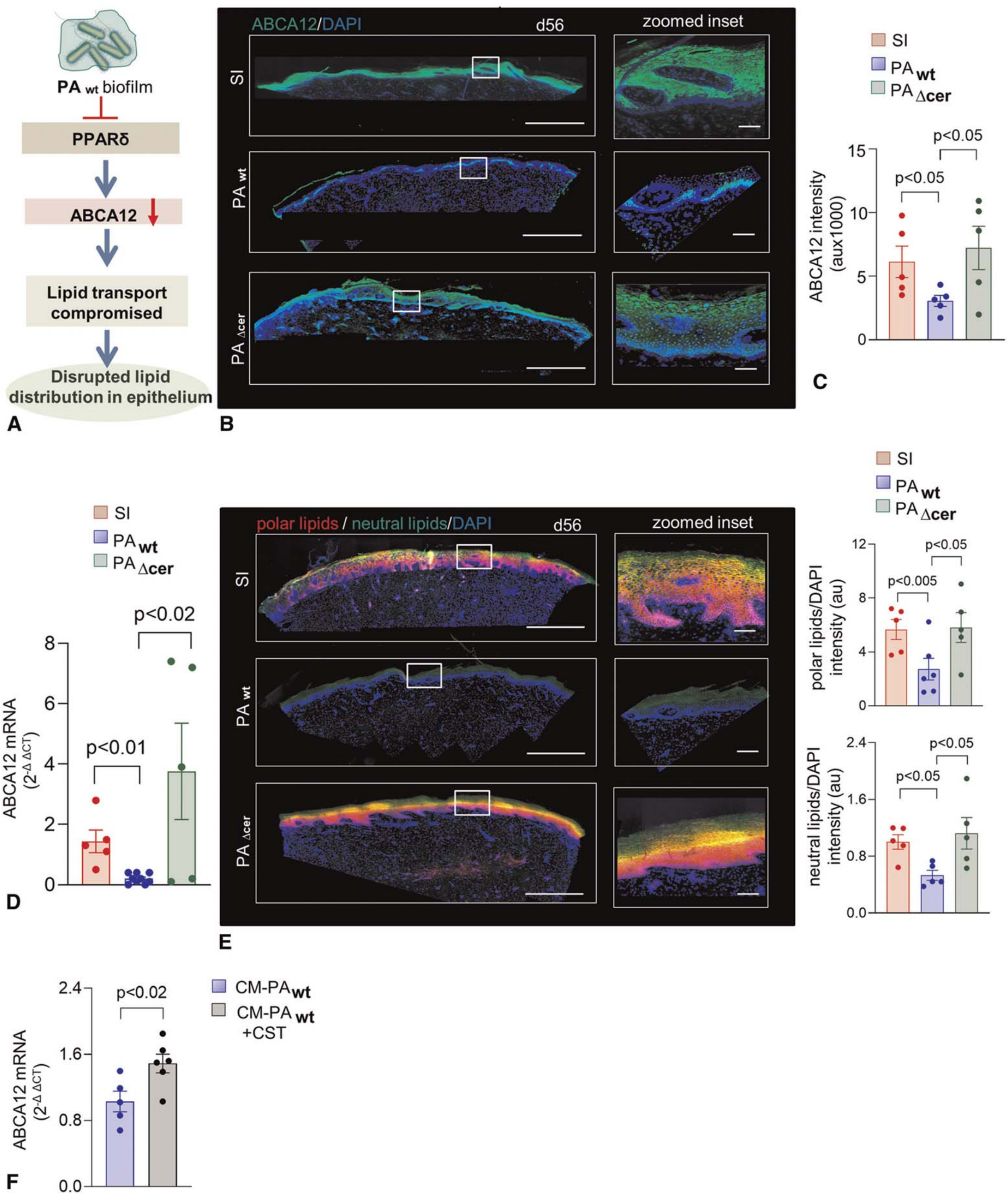

FIGURE 6. ABCA12 Expression Is Compromised Following PAwt Biofilm Infection. (A) Schematic depiction of the hypothetical pathway. (B and C) Loss of ABCA12 in wound-site epithelium which was infected with PA_{wt} compared to SI or $PA_{\Delta cer}$ groups. Scale bar = 500 μm with corresponding zoomed images of 50 μm. anti-ABCA12 (green) and DAPI (blue). Data presented as mean ± SEM (n = 5). (D) Downregulation of ABCA12 in porcine wounds infected with PA_{wt} compared to SI or $PA_{\Delta cer}$ wounds measured by qPCR. Whole tissue homogenate was used for analysis. Data presented as mean ± SEM (n = 5–8). (E) Disruption of neutral and polar lipid distribution at the affected wound-site skin. Scale bar = 500 μm with corresponding zoomed images of 50 μm. Nile Red staining with DAPI (blue) showing expression of polar lipids (red) and neutral lipids (green). Data presented as mean ± SEM (n = 5). (F) Ceramidastin (10 μg/mL) attenuated biofilm-induced loss of ABCA12 expression in human keratinocytes (HK) as measured by qPCR. Data presented as mean ± SEM (n = 5-6).

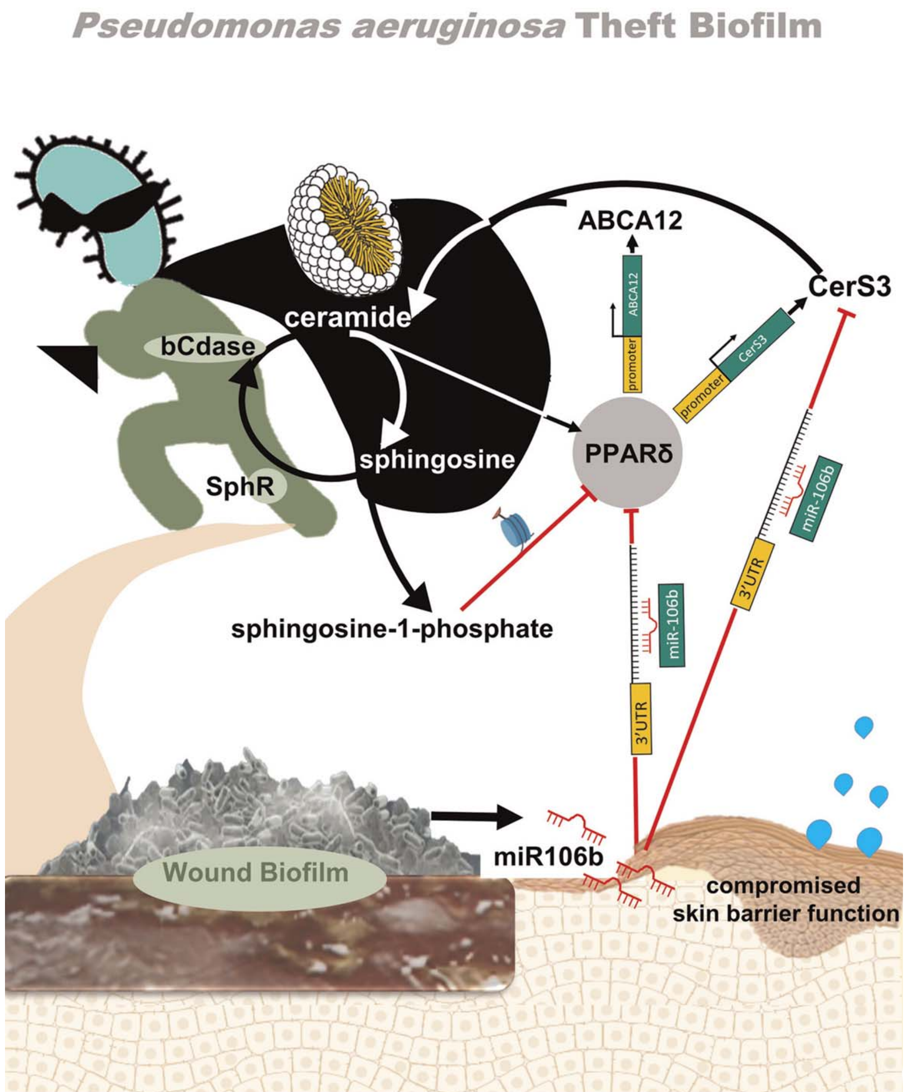

FIGURE 7. Summary figure. In cutaneous wounds, *Pseudomonas aeruginosa* forms pathogenic theft biofilm. Such biofilm severity relies on the theft of host lipids (ceramides) causing potent induction of bacterial ceramidase (bcdase). Skin ceramide biosynthesis is impaired by post-trancriptional gene silencing of CerS3 by biofilm-inducible miR106b. The limited ceramide pool, thus available as inducer of PPARδ, is threatened by elevated biofilm bcdase. Thus expression of PPARδ, a major regulator of skin lipid homeostasis, is blunted. PPARδ faces a second line of attack in the form of epi-genetic silencing by both biofilm-induc-ible miR–106b as well as S-1-P. Such loss of PPARδ compromised downstream genes including cutaneous lipid transporter ABCA12. Taken together, biofilm disrupts skin lipid homeostasis in a way that the site of wound repair cannot restore barrier function, a marker of functional wound healing.

Epidermal Lipid Transporter ABCA12 is Compromised Following Wound Biofilm Infection

Skin ceramides are responsible for an estimated 50% of all cutaneous lipids.[33] Other skin lipids play a significant role in enabling skin barrier function.[34] These 2 are known to interactively maintain skin health.[35] ABCA12, the transcription of which is PPARB-dependent (Fig. S9A-B, http://links.lww.com/SLA/D496), is an epidermal keratinocyte lipid transporter.[36] Wound-site ABCA12 expression was attenuated in response to biofilm infection by PA_{wt}, but not SI or PA_{DCer} (Fig. 6A-D, Fig. S9C, http://link-s.lww.com/SLA/D496). Such downregulation of ABCA12 was associated with overt changes in skin lipid distribution. At the wound-site epidermis, levels of polar and neutral lipids were sharply lower in response to biofilm infection by PA_{wt}, but not by SI or $PA_{\Delta Cer}$ (Fig. 6E). Mechanistic follow-up studies on human keratinocytes revealed that loss of ABCA12 in response to biofilm infection by PA_{wt} was prevented by ceramidastin demonstrating that the loss was caused by a breakdown product of keratinocyte ceramide acted upon by *bcdase* (Fig. 6F). Such finding was consistent with the prevention of loss of ABCA12 in response to infection by $PA_{\Delta Cer}$ (Fig. 6B and C). Fig. 7 presents a summary of mechanisms gleaned based on this work.

DISCUSSION

Armed with a wide range of lipid metabolizing inducible enzymes, PA is known to exploit host lipids to facilitate host cell binding and to evade host immune defenses.[37] In macrophages of the innate immune defense system, PA bolsters acid sphingomyelinase activity causing release of host lipid ceramides producing sphingo-lipid-rich rafts which helps internalization of PA.[38] In the lungs, PA lipoxygenase (pLoxA) oxidize host arachidonic acid–phosphatidyl-ethanolamine to cause bronchial epithelial ferroptosis and establish airway biofilm.[39] The notion that host lipids may be preemptively primed to compromise the ability of pathogenic microbes to co-opt them emerges as result. Induction of bacterial ceramidases by three orders of magnitude in PA biofilm-infected wound tissue, as reported in this work, called for a systematic investigation testing its significance in the healing response in biofilm affected wounds.

The Wound Healing Society recommends the study of porcine model as the most relevant preclinical model of skin wound heal-ing.[40] The present work is based on the study of an established model of chronic wound biofilm infection in immune-competent pigs in vivo.[1,2] The approach results in the establishment of an induced polymicrobial wound biofilm comprising of PA and *Acinetobacter baumannii.* Noninfected skin wounds colonized by normal skin flora of the porcine served as baseline control and were referred to as SI. Previous publications using this model revealed that PA establishes itself as the dominant strain as the wound becomes chronic.[1–3]

Skin serves the primary function of affording barrier defense. Loss of skin barrier increases vulnerability to infection and allergens.[41] Compromised skin barrier function is also associated with atopic dermatitis, psoriasis, contact dermatitis, and some specific genetic disorders.[42] Central to enabling the barrier function of skin are the skin ceramides.[12] Ceramide homeostasis of the skin depends on a dynamic balance between biosynthetic and catabolic pathways. The observation that biofilm infection by $PA_{wt,}$ but not $PA_{\Delta Cer}$, compromises barrier function of the repaired skin provides direct evidence implicating ceramide depletion in impaired restoration of barrier function during healing. CerS3 is recognized as the primary catalyst of long chain cutaneous ceramide synthesis.[30] This work recognizes biofilm-inducible miR–106b as a post-transcriptional gene silencer of CerS3.

Findings of this study demonstrate that PA leverages host lipids to bolster biofilm formation. Addition of cutaneous porcine lipid augmented biofilm formation in a manner sensitive to delipi-dation. Lipids *per se* were not the trigger because such augmentation of biofilm was absent with $PA_{\Delta Cer}$. Biofilm formation is linked with QS pathway of PA.[43] The present work demonstrates that ceramide breakdown products are capable of inducing *SphR.* During antibiotic resistance of PA, *SphR* is involved in quorum sensing VqSM-SphR interaction.[44] This pathway represents a plausible mechanism by which skin lipids may induce QS. In transcriptional regulation of host skin lipids, peroxisome proliferator-activated receptor PPARδ plays a central role.[45] This hub emerged as a central player in the paradigm unveiled by the findings of this study. Basal PPARδ activity in the skin is driven by ceramides.[31] The PA biofilm-dependent amplification loop, as described above, depleted skin ceramides thus lowering PPARδ activity. As part of that loop as ceramides were catabolized to sphingosine, S-1-P was produced. S-1-P epigenetically silenced PPARδ expression. It is known that QS molecule such 2-amino-acetophenone or other unrelated pathogen associated molecules of PA can regulate the host epigenome through HDAC1-mediated epigenetic reprogramming to enable tolerance of infection.[46] In lung injury secondary to inflammation caused by *PA,* ceramide-derived sphingosine and S1P are directly implicated.[47] Interestingly, cer-amide can also act as antimicrobials. Sphingosine effectively killed *S. aureus, Streptococcus pyogenes, Micrococcus luteus, Propioni-bacterium acnes, Staphylococcus epidermidis,* and moderately killed PA.[48] Sphingosine prevented and eliminated *Staphylococcus epider-midis* biofilm on orthopedic implant materials.[49] Sphingosine binds to bacterial membrane cardiolipin and limit growth.[50] Bacterial growth retardation is inherent to biofilm formation.[51] Consistently, the finding of this work reports that elevated S-1-P, a derivative of sphingosine, is associated with higher biofilm formation.

A third mechanism to downregulate PPARδ was contributed by PA biofilm inducible miR–106b. This may be viewed as a well-coordinated effort by PA biofilm to disable skin PPARδ and therefore hijack host metabolic processes to augment biofilm fate. Of particular interest is the observation that this entire cascade of events is triggered by host lipids. As it relates to the functional significance of PPARδ in barrier function of the skin, it is known that topical application of an agonist of PPARδ accelerates restoration of such function following injury.[52]

The ATP-binding cassette (ABC) transporter ABCA12, transcriptionally regulated by PPARδ, encodes a highly conserved group of proteins involved in active transport of a variety of lipids across biological membranes.[53] In epidermal keratinocytes, PPARδ upre-gulates ABCA12.[36] Loss of skin wound-site PPARδ in response to PA biofilm infection was associated with compromised ABCA12 expression. At the wound-site, where covering of the defect with repaired skin has been achieved it was noted that this ABCA12-deficient epidermis was also compromised in abundance of lipids. Such pathological manifestation has been also reported in congenital ichthyoses where low ABCA12 is associated with compromised skin barrier .[54] This is consistent with our previously report demonstrating that wounds with a history of biofilm infection appears closed but is not functionally closed as the site is deficient in barrier function.[1–3] Closed wound-site with a history of biofilm infection, featuring compromised barrier function, is known to biomechanically deficient with weak tensile strength.[3] This observation, taken together with the report that skin deficient in barrier function act as window allowing entry of pathogenic allergens to the body,[41] points towards the hypothesis that such affected site may be prone to wound recidivism and other threats to general health.[3]

In summary, this work reports that in the setting of cutaneous wound, pathogenic PA biofilm formation relies on the theft of host lipid factors which the bacteria use to turn on and sustain its bolstered ceramidase system which is otherwise weak. PA biofilm formation was highly responsive to its microenvironment such that in the context of skin wounds it utilized ceramide breakdown products to augment biofilm aggregates. This process was initiated by a massive induction of bacterial ceramidase in response to host lipids. Downstream products of such metabolism such as sphingosine and S-1-P were directly implicated in induction of ceramidase and inhibition of PPARδ, respectively. PA biofilm also silenced PPARδ *via* induction of miR–106b. Low PPARδ limits ABCA12 expression resulting in disruption of skin lipid homeostasis. Barrier function of the skin was thus compromised. The significance of such defect in the functional deficiency of the skin with respect to risk of infection and wound recurrence warrant further consideration.

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