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## Quorum sensing between bacterial species on the skin protects against epidermal injury in atopic dermatitis

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**Author contributions:** M.R.W. wrote the manuscript and was responsible for designing and conducting the majority of the experiments. S.K.C. and A.L.C. developed the *S. epidermidis* RP62A autoinducing peptide knockout strain L.S.Z. and K.Z. analyzed the AD metagenomic data. S.K. was responsible for helping with murine experiments and determination of *S. aureus psm $\alpha$*  expression. D.A.T., H.L.W., and N.B.C. determined the structure of *S. hominis* C5 autoinducing peptide. J.A.S., A.M.O., and M.C.L. helped with protein and mRNA expression experiments, while J.A.S. helped with RNA-seq analysis. T.N. provided project guidance and ideas and isolated the clinical CoNS strains used in this study. A.R.H. provided the *S. aureus* reporter strains used in this study as well as project guidance. R.L.G. conceived and interpreted experiments, wrote the manuscript, and supervised all aspects of this study.

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Materials and Methods

Fig. S1. *S. aureus* PSM $\alpha$  changes essential barrier gene and cytokine expression in human keratinocytes.

Fig. S2. *S. aureus* PSM $\alpha$  and proteases are responsible for barrier damage and induction of inflammation on murine skin.

Fig. S3. Effect of AD skin disease severity on *S. epidermidis agr* type I to *S. aureus* relative abundance by body site.

Fig. S4. Clinical CoNS isolates inhibit *S. aureus agr* activity without affecting growth.

Fig. S5. *S. hominis* C5 live bacteria specifically inhibit *S. aureus*-mediated barrier damage and inflammation.

Fig. S6. *S. hominis* C5 inhibits *S. aureus agr* types I to III but not type IV.

Fig. S7. *S. hominis* C5 autoinducing peptide inhibits *S. aureus*-driven inflammation and barrier damage.

Table S1. Bacteria strains and plasmids used.

Table S2. Primers used for quantitative polymerase chain reaction and allelic replacement. Data file S1. Raw data (available as Excel file).

References (44–56)

**Competing interests:** M.R.W. and R.L.G. are co-inventors of UCSD technology related to the bacterial quorum sensing inhibition therapy discussed here under patent PCT/US18/49237 titled “Molecular bacteriotherapy to control skin enzymatic activity.” R.L.G. is a co-founder, scientific advisor, consultant, and has equity in MatriSys Biosciences, and is a consultant, receives income from, and has equity in Sente.

**Data and materials availability:** All data associated with this study are present in the paper or Supplementary Materials. RNA-seq data are available through the NCBI Gene Expression Omnibus (GEO) online data repository under accession number GSE124818. Bacterial genome sequences are available at NCBI BioProject under accession PRJNA514867. Primary data are reported in data file S1.

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## Abstract

Colonization of the skin by *Staphylococcus aureus* is associated with exacerbation of atopic dermatitis (AD), but any direct mechanism through which dysbiosis of the skin microbiome may influence the development of AD is unknown. Here, we show that proteases and phenol-soluble modulins (PSM $\alpha$ ) secreted by *S. aureus* lead to endogenous epidermal proteolysis and skin barrier damage that promoted inflammation in mice. We further show that clinical isolates of different coagulase-negative staphylococci (CoNS) species residing on normal skin produced autoinducing peptides that inhibited the *S. aureus agr* system, in turn decreasing PSM $\alpha$  expression. These autoinducing peptides from skin microbiome CoNS species potently suppressed PSM $\alpha$  expression in *S. aureus* isolates from subjects with AD without inhibiting *S. aureus* growth. Metagenomic analysis of the AD skin microbiome revealed that the increase in the relative abundance of *S. aureus* in patients with active AD correlated with a lower CoNS autoinducing peptides to *S. aureus* ratio, thus overcoming the peptides' capacity to inhibit the *S. aureus agr* system. Characterization of a *S. hominis* clinical isolate identified an autoinducing peptide (SYNVCGGYF) as a highly potent inhibitor of *S. aureus agr* activity, capable of preventing *S. aureus*-mediated epithelial damage and inflammation on murine skin. Together, these findings show how members of the normal human skin microbiome can contribute to epithelial barrier homeostasis by using quorum sensing to inhibit *S. aureus* toxin production.

## INTRODUCTION

Atopic dermatitis (AD) is among the most common immune disorders and poses a serious risk of comorbidities and a major burden to patient quality of life (1,2). Risk of developing AD is increased in patients with genetic defects in skin barrier function and is also associated with early life environmental exposure to various allergens (3–5). Furthermore, recent studies of the composition of the skin microbial community have suggested that a relative abundance of bacteria such as *Staphylococcus aureus* and coagulase-negative staphylococcal (CoNS) species may predict the development of AD (6, 7). These observations follow several decades of reports that *S. aureus* often colonizes lesions on the skin of patients with AD (8) and positively correlates with disease severity (9–11). Despite the large body of work to identify genetic, environmental, and microbial risk factors that may cause AD, there is as of yet no validated, cohesive hypothesis to link these observations together into a unifying pathophysiologic mechanism.

AD is characterized by a T helper 2 (T<sub>H</sub>2)-dominant immune phenotype. Patients with AD have increased amounts of T<sub>H</sub>2 cytokines such as interleukin-4 (IL-4) and IL-13 in the skin (12, 13). These cytokines promote decreased function of the skin barrier by inhibiting expression of filaggrin (14) and suppressing expression of antimicrobial peptides such as cathelicidin and  $\beta$ -defensin-2. These defects promote dysbiosis of the skin bacterial community and enhanced colonization by *S. aureus* (15). Therapy targeting the IL-4 receptor  $\alpha$  results in a substantial improvement in disease (16). The strong association between T<sub>H</sub>2 cytokine activity, barrier function, antimicrobial activity, and disease outcome supports

efforts to define a causal link between these essential epidermal functions. However, it has not been shown how dysbiosis can promote or enable skin disease.

Recent evidence has demonstrated that virulence factors produced by *S. aureus*, such as phenol-soluble modulins (PSMs) and PSM $\delta$ , can promote skin inflammation in mice (17–20). PSMs represent a family, often cytolytic, of peptides secreted by *S. aureus* and include PSM $\alpha$ 1 to PSM $\alpha$ 4, PSM $\beta$ 1 and PSM $\beta$ 2, PSM $\delta$  and the recently observed PSM-mec. In addition to promoting inflammation, *S. aureus* virulence factors can also cause epidermal barrier disruption, a key element in the pathophysiology of AD, by inducing expression of endogenous proteases from keratinocytes (21). Other studies have shown that the potential for *S. aureus* to induce inflammation can be linked to genetic disorders in barrier assembly including mutations in the filaggrin gene and the penetration of bacteria into deeper layers of the skin (22, 23). Together, we hypothesized that skin inflammation is promoted by penetration of *S. aureus* below the epidermis and that this may be caused by the action of *S. aureus* to increase protease activity in keratinocytes, thus disrupting the skin barrier.

This investigation sought to identify the molecular mechanism responsible for the deleterious effects of *S. aureus* on the epidermal barrier and further define how dysbiosis at the skin surface permits this microbe to promote inflammation. Our study uncovers a previously unappreciated interaction between microbial communities on the skin that reinforces the need for microbial diversity in AD. These data show that interspecies quorum sensing between bacteria on human skin is an important defense mechanism for suppressing the capacity of *S. aureus* to damage the epidermis.

## RESULTS

### PSM $\alpha$ and proteases produced by *S. aureus* induce epidermal barrier damage

To understand the potential role of *S. aureus* PSMs on epidermal barrier function, we assessed normal human epidermal keratinocytes (NHEKs) for their capacity to express proteolytic activity when exposed to *S. aureus* PSMs. Treatment with conditioned medium from wild-type *S. aureus* (USA300 LAC) or the same strain with targeted deletions in either the *psma* or *psm $\beta$*  operons revealed that *psma* was required for induction of trypsin-like serine protease activity and increased mRNA expression of kallikrein 6 (*KLK6*) (Fig. 1, A and B). We next tested synthetic PSM $\alpha$ 1 to PSM $\alpha$ 4 and PSM $\beta$ 2 peptides that we synthesized on the basis of the genes encoded within the *psma* and *psm $\beta$*  operons and found that all PSM $\alpha$  peptides could stimulate trypsin activity, whereas PSM $\beta$ 2 could not (Fig. 1C). PSM $\alpha$ 3, the strongest inducer of protease activity in NHEKs, induced trypsin activity and *KLK6* mRNA expression in NHEKs in both a dose- and time-dependent manner and also induced cytokine production [IL-6, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and IL-1 $\alpha$ ] in human keratinocytes (fig. S1, A to D). Furthermore, transcriptional profiling by RNA sequencing (RNA-seq) of NHEKs exposed to PSM $\alpha$ 3 showed that this toxin had a broad effect on expression of several genes related to the skin barrier, including multiple proteases [*KLKs* and matrix metalloproteinases (*MMPs*)], components of the physical barrier (filaggrin, desmoglein-1, loricrin, involucrin, and keratins), antimicrobial peptides, and proinflammatory cytokines (Fig. 1, D and E, and fig. S1E). Deletion of *psma*, but not *psm $\beta$* , also reduced the production of IL-6, TNF $\alpha$ , and IL-1 $\alpha$  from NHEKs (fig. S1, F to H).

To validate the role of the *psma* operon on the epidermal barrier in vivo, mice were colonized on the skin surface for 72 hours with equal numbers of the *S. aureus* USA300 LAC wild type or the corresponding *psma* mutant strain. *S. aureus* induced erythema, scaling, and epidermal thickening of murine skin in a *psma*-dependent manner (Fig. 1F). Despite increased epidermal thickness, we observed an increase in TEWL, a well-established method to assess skin barrier damage after exposure to wild-type *S. aureus*, but not when *psma* was absent (Fig. 1G). Skin barrier disruption of a fully differentiated epidermis in vivo was also dependent on the expression of protease activity by *S. aureus*. Using a mutant strain of *S. aureus* derived from the wild-type USA300 LAC (AH1263) parent strain that lacks all 10 major secreted proteases (AH1919) (24), we showed decreased injury and improved TEWL when *S. aureus* lacked protease activity, despite fully intact *psma* expression (Fig. 1, F and H). An increase in epidermal trypsin activity, epidermal thickness, *Klk6*, and *Ii6*, *Ii7a*, and *Ii7f* transcript expression was observed in mice exposed to wild-type *S. aureus*, but not when exposed to *psma* or protease-deficient strains (fig. S2). A decreased inflammatory response to the protease-deficient mutant contrasted with the response of cultured NHEKs that showed only minor differences in endogenous protease activity induction (21) and no change to *KLK6* expression or cytokine (IL-6, TNF $\alpha$ , and IL-1 $\alpha$ ) responses when conditioned medium from the protease-deficient mutant was applied (fig. S1, I and J). This suggests that *S. aureus* protease activity is necessary for *psma* to activate keratinocytes when an intact stratum corneum is present. Despite changes in the skin barrier and inflammatory milieu, *S. aureus* survival on skin was not influenced by deletion of *psma* or protease activity (Fig. 1, I and J). Together, these data show that both production of PSM $\alpha$  peptides and protease activity from *S. aureus* result in damage to the epidermal barrier, a critical step in promoting the skin inflammation seen in AD

### ***Staphylococcus epidermidis* autoinducing peptide inhibits *S. aureus agr* activity**

Both the *S. aureus* PSM $\alpha$  peptides and secreted proteases are under regulation of the accessory gene regulatory (*agr*) quorum sensing system. *S. aureus* clinical isolates have been found to have four distinct *agr* types, with *agr* type I being the most prominent in AD subjects (25, 26). A *S. epidermidis agr* type I laboratory isolate was previously revealed to produce an autoinducing peptide that could inhibit *S. aureus agr* types I to III but not type IV, but little is known of the influence of other *agr* types or the skin commensal community as a whole on *S. aureus agr* activity (27). To investigate this, conditioned culture supernatants from defined laboratory strains of *S. epidermidis* with *agr* types I (RP62A), II (1457), or III (8247) were first tested with a *S. aureus* USA300 LAC *agr* type I P3-YFP reporter strain to explore whether *S. epidermidis agr* activity could influence the *S. aureus agr* system. *S. epidermidis agr* type I inhibited *S. aureus agr* activity, whereas *S. epidermidis agr* types II and III had little effect (Fig. 2, A and B). Targeted deletion of the *S. epidermidis* (RP62A) *agr* type I autoinducing peptide within the *agrD* gene region abolished the capacity of *S. epidermidis* to inhibit *S. aureus agr* activity (Fig. 2C). Furthermore, we observed that the increase in NHEK trypsin activity was inhibited when *S. aureus* was cultured in the presence of supernatant from wild-type *S. epidermidis agr* type I, but not by *S. epidermidis* lacking this autoinducing peptide (Fig. 2D). These experiments demonstrate that some *S. epidermidis* laboratory strains could inhibit the capacity of *S. aureus* to induce keratinocyte trypsin activity and may therefore be relevant to inflammation in AD

## CoNS prevent *S. aureus* damage to AD skin

Having established the potential for a *S. epidermidis agr* type I strain to decrease the capacity of *S. aureus* to induce protease activity in human keratinocytes, we next investigated the abundance of *S. epidermidis agr* types on patients with AD. We analyzed previously published metagenomic data available from the skin microbiome of 5 healthy subjects and 11 subjects with AD (28). Of the 60 *S. epidermidis* strains detected on AD skin, the most frequent *agr* type was that of *agr* type I (Fig. 2E). Data were next stratified on the basis of severity [objective SCORing Atopic Dermatitis (oSCORAD)], and results were pooled from seven body sites. Comparison of the abundance of *S. epidermidis agr* type I to *S. aureus* showed that *S. epidermidis agr* type I became relatively less abundant in AD subjects with increased disease severity across all body sites (Fig. 2, F and G). Analysis by body site showed a similar trend at all locations, with the most frequent affected sites of the antecubital crease and popliteal crease showing the most notable association with global severity (fig. S3). These observations suggested that the relative abundance of *S. epidermidis agr* type I compared to *S. aureus* in the AD skin microbiome is associated with clinical disease. This association is consistent with a potential quorum sensing mechanism that would require an increased ratio of autoinducing peptides from *S. epidermidis* to inhibit *S. aureus* disease-promoting protease and toxin production.

Having established the function of a laboratory strain of *S. epidermidis* to inhibit the capacity of *S. aureus* to damage the epidermis, and observing that there was a lack of *S. epidermidis agr* type I strains relative to *S. aureus* in more severe AD, we next sought to determine whether quorum sensing interactions might exist between other members of the skin microbiome and *S. aureus*. We screened multiple clinical CoNS isolates from AD and healthy subjects for the capacity of culture supernatants from these isolates to inhibit *S. aureus agr* activity. This experiment revealed that diverse species other than *S. epidermidis*, including *Staphylococcus hominis*, *Staphylococcus warneri*, and *Staphylococcus capitis*, have potent inhibitory activity against *S. aureus* and encode putative autoinducing peptides (Fig. 2, H and I). We screened 144 clinical CoNS isolates from healthy individuals and 288 isolates from both nonlesional and lesional AD skin swabs and found that the frequency of CoNS strains that could inhibit *S. aureus agr* activity was similar across all subjects regardless of disease state (fig. S4, A and B). As CoNS from healthy skin often have the capacity to inhibit growth of *S. aureus* (29), culture supernatant was used at a 25% dilution that effectively inhibited *agr* but did not inhibit *S. aureus* growth (fig. S4C). These findings suggest that multiple diverse members of the skin microbial community may contribute to suppressing *S. aureus agr* activity.

To establish whether quorum sensing interactions between CoNS and *S. aureus* can occur on live skin, *S. aureus agr* activity was assessed on mice by In Vivo Live Imaging (IVIS) using the *S. aureus* USA300 LAC *agr* type I P3-Lux promoter strain. We chose *S. hominis* C5 as a model clinical CoNS isolate for the remainder of this study as this strain potently inhibited *S. aureus agr* type I activity but did not have antimicrobial activity against *S. aureus*, thus permitting accurate assessment of *agr* activity. Application of live *S. hominis* C5 together with *S. aureus* to mice at a 1:1 ratio resulted in potent inhibition of *agr* activity, inflammation, TEWL, trypsin activity, and decreased *Klk6* and cytokine expression (Fig. 3,

A to G). At a higher *S. aureus* to *S. hominis* ratio (10:1), the protective activity of *S. hominis* was lost (fig. S5, A and B). This was similar to the increase in AD severity seen in subjects with an increased ratio of *S. aureus* (Fig. 2F). Use of a clinical CoNS isolate that did not inhibit *agr* activity (*S. epidermidis* A11) did not prevent the capacity of *S. aureus* to promote barrier damage and inflammation (fig. S5, C and D). Co-application of *S. hominis* C5 did not decrease the abundance of *S. aureus* (fig. S5, B and D).

Sequence analysis of metagenomic data from patients with AD revealed that there was diversity in *S. aureus agr* types on patients with AD, with the most common being *agr* types I and II (fig. S6). We sought to determine whether *S. aureus* clinical isolates had an *agr* type allowing them to express PSM $\alpha$  peptides and induce damage to the epidermis similar to the laboratory isolate USA300 LAC. We screened skin swabs from healthy, nonlesional, and lesional patients with AD. The *S. aureus psm $\alpha$*  operon transcript was much more abundant in samples from patients with AD (Fig. 3H). *S. aureus* PSM $\alpha$ 3 protein deposition was detected in the epidermis of lesional AD skin sections by immunohistochemistry (Fig. 3I). Functional screening of 11 distinct clinical *S. aureus* isolates obtained from the lesional skin of 11 subjects with AD demonstrated that all of these strains were able to induce trypsin activity in NHEKs, and that the mRNA expression of the *psm $\alpha$*  operon correlated with the bacterial capacity to induce trypsin activity (Fig. 3J). *S. hominis* C5 supernatant inhibited *S. aureus agr* types I to III, but not type IV, and all of the 11 *S. aureus* isolates from AD subjects were identified as being *agr* type I or II (fig. S6). We found that *S. hominis* C5 could also prevent PSM $\alpha$ 3 protein expression in a potent *S. aureus* AD isolate (AD 38) (Fig. 3K). Last, co-colonization of murine back skin with AD 38 and live *S. hominis* C5 (1:1 ratio) was shown to inhibit skin injury and inflammation without changing *S. aureus* abundance (Fig. 3, L to N). This treatment also prevented AD 38–induced trypsin activity, *Klk6* expression, and cytokine expression (fig. S5, E to G). Overall, these data confirm expression of PSM $\alpha$  on AD skin and support the clinical relevance of our observations that CoNS can inhibit the *agr* activity and prevent *S. aureus* virulence factor production.

### Purification of an autoinducing peptide from *S. hominis* and confirmation of activity

To further validate the conclusion that *S. aureus agr* inhibition was due to autoinducing peptide expression from the microbiome of human skin, we next identified the autoinducing peptide from the *S. hominis* C5 clinical isolate. This was accomplished by several initial biochemical isolation steps from crude *S. hominis* C5 supernatant followed by liquid chromatography–mass spectrometry (LC-MS) of enriched fractions. Our purification and sequence analysis detected an autoinducing peptide sequence (S-Y-N-V-C-G-G-Y-F) with a cyclic ring between the C and F amino acids (Fig. 4, A to C). The identified sequence was consistent with an autoinducing peptide in the *agrD* coding region predicted by genomic DNA sequencing (Fig. 2I).

To validate that this *S. hominis* C5 autoinducing peptide could inhibit *S. aureus agr* activity, we generated a synthetic peptide and added it to the *agr* reporter strain. The synthetic autoinducing peptide potently inhibited *S. aureus agr* activity in a dose-dependent manner and showed a median inhibitory concentration (IC<sub>50</sub>) of 0.6243 nM (Fig. 4D). The synthetic autoinducing peptide also inhibited the capacity of this *S. aureus* strain to induce trypsin

activity in NHEKs (Fig. 4E). When the synthetic autoinducing peptide was directly added to mouse skin colonized with *S. aureus*, *agr* activity was inhibited (Fig. 4, F and G). The synthetic autoinducing peptide also inhibited TEWL, *Klk6*, and cytokine expression induced on murine skin by *S. aureus* without inhibiting *S. aureus* abundance (Fig. 4, H to J, and fig. S7). These data confirm that an autoinducing peptide from a CoNS species resident on human skin can inhibit the *agr* activity of *S. aureus*, supporting the conclusion that interspecies quorum sensing mechanisms in the skin microbiome can protect against epidermal damage promoted by *S. aureus*.

## DISCUSSION

In this study, we provided two interrelated mechanisms that shed new light on the development and control of AD. Our findings show that *S. aureus* PSM $\alpha$  peptides directly act on human keratinocytes, the main cell type responsible for establishment of the physical barrier of the skin. Exposure to PSM $\alpha$  leads to increased proteolytic activity in the epidermis, breakdown of the epidermal barrier, and subsequent skin inflammation. This observation explains how *S. aureus* residing on the skin surface can promote and exacerbate disease in the absence of the deep tissue invasion that defines infection. The second main finding from this study is based on earlier observations that PSM $\alpha$  is under control of the quorum sensing *agr* system (30). As autoinducing peptides from some laboratory bacterial species have been found to repress the *S. aureus agr* system (27, 31, 32), we hypothesized that such an interspecies interaction within the human skin microbiome may be a normal event to maintain homeostasis even if colonization with *S. aureus* can occur. Our data demonstrated that several different CoNS species, but not all strains within these species, encode autoinducing peptides that repress *S. aureus agr* activity. We showed that a beneficial CoNS strain or the synthetic CoNS autoinducing peptide was capable of inhibiting skin inflammation induced by *S. aureus*. We found that the stoichiometry of these interspecies interactions is unfavorable during disease flares in patients with AD, potentially indicating that when dysbiosis becomes extreme, these subjects then become exposed to PSM $\alpha$ . This is consistent with the direct observation of PSM $\alpha$  mRNA and protein deposition on lesional AD skin, an event that would not occur if CoNS autoinducing peptides were sufficiently abundant to suppress PSM $\alpha$  expression. Together, these observations show a previously unrecognized mechanism by which the diverse members of the skin microbiome can inhibit the disease-promoting activity of *S. aureus*. This observation could potentially explain why loss of bacterial diversity from excess abundance of *S. aureus* is strongly associated with increased disease severity.

*S. aureus*-secreted proteases, including the V8 protease, can directly cause skin barrier damage in mice (33) and play a vital role for *S. aureus* penetration into deeper layers of the epidermis and dermis, an event that triggers maximal induction of T<sub>H</sub>2 and T<sub>H</sub>17 inflammatory responses in murine AD models (22, 23). In this study, we found that *S. aureus* subverted the healthy murine epidermal barrier by the combined action of protease activity and production of PSM $\alpha$  peptides. *S. aureus*-secreted proteases appeared essential to enable PSM $\alpha$  peptides to induce inflammation in vivo, where an intact stratum corneum protects underlying live keratinocytes. In vitro, *S. aureus* proteases had little to no effect on human keratinocytes, whereas PSM $\alpha$  expression was the major factor responsible for



inducing keratinocyte trypsin activity. This suggests that *S. aureus*-secreted proteases are required for *S. aureus* to penetrate the skin in order for PSM $\alpha$  to stimulate keratinocytes. This process of inter-kingdom signaling explains how minor colonization by *S. aureus* could be amplified to promote major defects in the skin barrier. Future work is needed to better understand how *S. aureus* can penetrate the epidermal barrier including analyzing exactly what contribution each of the 10 known secreted *S. aureus* proteases including aureolysin, V8, staphopain A/B, and SplA-F have in inducing skin barrier damage.

We confirmed previous reports that PSM $\alpha$  peptides could induce an array of proinflammatory cytokines, including IL-6, IL-1 $\alpha$ , and TNF $\alpha$ , in human keratinocytes and skin. Keratinocyte secretion of IL-1 type cytokines was recently shown to be important for subsequent T<sub>H</sub>17 responses in murine skin (17, 18). T<sub>H</sub>17 responses have also been found to be clinically important in acute AD (34–36) and may explain an important component of how both *S. aureus agr*-regulated toxins such as PSM $\alpha$  and secreted proteases stimulate skin inflammation. Previous work has shown that a more T<sub>H</sub>2-dominant response in mice is observed after *S. aureus* colonization of ovalbumin-sensitized mice lacking flaggrin (22). This further supports the important interplay between environment, host genotype, and *S. aureus* colonization to drive human AD.

Here, we validated that the *S. epidermidis agr* type I autoinducing peptide was active against *S. aureus* USA300 LAC *agr* type I and protective for human keratinocytes, whereas *S. epidermidis agr* types II and III were not active. Analysis of clinical samples from AD subjects demonstrated the presence of these strains on human skin and suggested that disease flare severity correlates with a decrease in the abundance of *S. epidermidis agr* type I relative to *S. aureus*, a condition that would promote PSM $\alpha$  expression and disease progression. One limitation of the current study is that the absolute abundance of autoinducing peptides and production of PSM $\alpha$  peptides were not measured during the course of disease flares. Such information would provide important supporting evidence that the quorum sensing interaction causes disease or promote remissions. In addition, despite the benefit displayed in this study by restoring commensal CoNS strains to the diseased skin phenotype to prevent *S. aureus agr* activity, more work is necessary to use this treatment as a potential therapeutic option. Several studies have eluded to biofilm formation being inversely regulated by the *S. aureus agr* system, with biofilms promoted by inhibition of *agr* (37, 38). Biofilms have been detected on subjects with AD (39), and their presence increases resistance of *S. aureus* to killing by host antimicrobials such as LL-37 (40). Therefore, although CoNS autoinducing peptides appear to be protective against skin inflammation induced by *S. aureus*, it is also possible that this promotes persistent colonization. This study also did not examine the efficacy of transplanted autoinducing peptide inhibitory CoNS strains to survive on diseased skin, or the stability of the synthetic CoNS autoinducing peptide on the normal skin surface. Understanding the efficacy of longterm application of the synthetic autoinducing peptides or CoNS application onto epidermal models is essential for human therapeutic application.

The data from the current study illustrate the complexity of interspecies and inter-kingdom communication on human skin by showing how multiple clinical CoNS isolates could prevent the capacity of *S. aureus* to induce skin barrier damage and inflammation. We

showed that most, but not all, *S. aureus* colonies could induce epidermal damage. We also showed that the presence of live clinical CoNS isolates, or a synthetic autoinducing peptide identified in one strain, was protective against clinical *S. aureus* strains expressing PSM $\alpha$ . It is possible that additional, unknown chemical signals that regulate *S. aureus agr* activity may also exist on human skin

Together, we provide evidence that quorum sensing between some commensal CoNS species and *S. aureus* on human skin acts in a beneficial manner by inhibiting the negative consequences of *S. aureus* toxin production. This protective mechanism may act in parallel with the capacity of some CoNS strains to directly inhibit *S. aureus* survival (29). Because autoinducing peptide action does not limit *S. aureus* growth, evolutionary pressure to avoid *agr* inhibition is much different than the selective pressure for *S. aureus* to evolve antimicrobial resistance. By using both autoinducing peptide and antimicrobial strategies to compete with *S. aureus*, such CoNS strains could become a highly desirable member of the healthy human skin microbiome. These findings suggest that the pathophysiology of AD requires better knowledge of the failure of host skin to support these mutualistic relationships over the disease-promoting activity of *S. aureus*. Understanding how to optimize this may lead to improvements to existing therapeutic strategies.

## MATERIALS AND METHODS

### Study design

The objective of this study was to determine how components of the microbiome including *S. aureus* strains and CoNS species and strains promote or protect against epidermal damage in AD. This was done using commercial primary human keratinocytes, epicutaneous murine models, and bacteria harvested from human skin and through computational analysis of published skin microbiome sequencing. All experiments in this study using in vitro and in vivo techniques were carried out with a minimum  $n = 3$  for biological replicates. Sample analysis was performed quantitatively, without assessment of power, in an unblinded manner and confirmed by at least two to three independent experiments as indicated in the figure legends. Primary data are reported in data file S1

### Collection of bacteria from human subjects

All experiments involving human subjects were carried out according to protocols approved by University of California, San Diego (UCSD) Institutional Review Board (project no. 071032). Skin swabs and bacteria isolates from healthy volunteers and patients with AD used in this study were collected from a previous study at UCSD under the protocol above with prior patient consent (29). Briefly, collection of surface bacteria was done from a premeasured area (15 cm<sup>2</sup>) of lesional skin on the antecubital crease of subjects with AD. Swabs were immediately placed into a 3% tryptic soy broth (TSB) and 16.67% glycerol solution and frozen at  $-80^{\circ}\text{C}$ . For collection of clinical *S. aureus* and CoNS isolates, swabs were rapidly thawed, vortexed, and serially diluted onto mannitol salt agar selection plates supplemented with 3% egg yolk. *S. aureus* was distinguished from CoNS according to mannitol metabolism and the egg yolk reaction as described previously, and isolates were stored at  $-80^{\circ}\text{C}$  in the same solution as above for further analyses (29, 41). Confirmation of

species of select CoNS strains was identified by full-genome sequencing analysis as indicated below.

### Bacterial preparation

All bacteria used in this study are listed in table S1. All staphylococci (*S. aureus* and CoNS strains) were grown overnight (18 hours) to stationary phase in 3% TSB at 300 rpm in a 37°C incubator unless stated otherwise. This growth time for all staphylococci indicated about an OD<sub>600nm</sub> reading of 10 and  $1 \times 10^9$  CFU. Specific strains were grown with antibiotic selection where indicated in table S1 at the following concentrations: Erm (5 µg/ml), Lcm (25 µg/ml), and Cm (10 µg/ml). For the treatment of bacterial supernatant on human keratinocytes or murine skin, bacteria cultured overnight were pelleted (15 min, 4000 rpm, room temperature) followed by filtered sterilization of the supernatant (0.22 µm). For mouse experiments with live bacteria colonization, bacterial CFU was approximated by OD<sub>600nm</sub> before application to mouse back skin followed by confirmation of the actual CFU the following day.

### NHEK culture

NHEKs (Thermo Fisher Scientific) were cultured in EpiLife medium containing 60 µM CaCl<sub>2</sub> (Thermo Fisher Scientific) supplemented with 1 EpiLife Defined Growth Supplement (Thermo Fisher Scientific) and 1× antibiotic-antimycotic [penicillin (100 U/ml), streptomycin (100 U/ml), amphotericin B (250 ng/ml); Thermo Fisher Scientific] at 37°C, 5% CO<sub>2</sub>. NHEKs were only used for experiments between passages 3 and 5. For experiments, NHEKs were grown to 70% confluency followed by differentiation in high-calcium EpiLife medium (2 mM CaCl<sub>2</sub>) for 48 hours to simulate the upper layers of the epidermis. For bacterial supernatant treatments, differentiated NHEKs were treated with sterile-filtered bacterial supernatant at 5% by volume to EpiLife medium for 24 hours. Similarly, for synthetic PSM treatments, the peptides (5 to 50 µg/ml) were added to the NHEKs for 24 hours in dimethyl sulfoxide (DMSO).

### *S. aureus* epicutaneous mouse model

Eight-week-old male C57BL/6 (The Jackson Laboratory) mice were used for all experiments ( $n = 5$  to 6 per condition), as specified in the figure legends. Mice were co-housed with  $n = 3$  to 5 per cage for all experiments. All animal experiments were approved by the UCSD Institutional Animal Care and Use Committee (protocol no. S09074). Mouse hair was removed by shaving and applying Nair for 2 min followed by immediate removal with alcohol wipes. The skin barrier was allowed to recover from hair removal for 48 hours before application of bacteria. *S. aureus* ( $1 \times 10^7$  CFU) in 3% TSB was applied to murine skin for 48 to 72 hours at a 100-µl volume on a 2-cm<sup>2</sup> piece of sterile gauze. Tegaderm was applied on top of gauze to hold in place for the duration of the treatment. For *S. aureus agr* inhibition experiments, live *S. hominis* C5 (1:1) or synthetic autoinducing peptides (10 µg) were combined with *S. aureus* in 3% TSB immediately before application on gauze.

### Synthetic PSM preparation

All synthetic PSMs were produced by LifeTein. Peptides were produced at 95% purity with N-terminal formylation. PSM sequences were as follows:

PSM $\alpha$ 1, fMGIIAGIHKVIKSLIEQFTGK; PSM $\alpha$ 2, fMGIIAGIHKFIKGLIEKFTGK;

PSM $\alpha$ 3, fMEFVAKLFFKFFKDLLGKFLGNN; PSM $\alpha$ 4, fMAIVGTIHKIKAIIDIFAK;

PSM $\beta$ 2, fMTGLAEAIANTVQAAQQHDSVKLGTSIVDIVANGVLLGKLFGE.

Peptides were resuspended in DMSO and concentrated by SpeedVac into 500 mg of powder stocks stored at  $-80^{\circ}\text{C}$  before reconstitution in DMSO for experiments.

### *S. hominis* C5 autoinducing peptide structure determination

We confirmed the structure of *S. hominis* C5 autoinducing peptide as S-Y-N-V-c(C-G-G-Y-F) (calculated  $m/z$ , 991.3984) as follows. *S. hominis* C5 was cultured for 24 hours in 3% TSB medium and was partially purified using solid-phase extraction as follows. The bacteria sterile-filtered (0.22  $\mu\text{m}$ ) spent medium was inoculated with 80% ammonium sulfate for 1 hour followed by centrifugation and resuspension of the pellet in water. The fraction was further filtered using size-exclusion centrifugation and by collecting the <3-kDa filtrate followed by running the filtrate through a Sep Pak C18 column (Waters) collecting a 40% acetonitrile elution after discarding the flow through and 20% acetonitrile fractions. The 40% acetonitrile- eluted fraction was dried on a SpeedVac and resuspended in water. This partially purified fraction from the *S. hominis* C5 spent medium and medium-only control was analyzed in the positive ion mode using an Acquity ultra-performance liquid chromatography (UPLC) system (Waters Corp.) coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). The medium control and *S. hominis* C5 fraction were injected directly into the UPLC-MS system. Samples were analyzed using a 7.5- $\mu\text{l}$  injection volume and a 10-min acetonitrile/water gradient as described previously (42). MS-MS data were collected by subjecting the  $[\text{M} + \text{H}]^{+}$ -ion  $m/z$  991.3984 to higher-energy collisional dissociation (HCD) fragmentation with a normalized collision energy of 25. The *S. hominis* C5 autoinducing peptide sequence from the spent medium was determined to be S-Y-N-V-c(C-G-G-Y-F) and was synthesized by AnaSpec at 90% purity by high-performance liquid chromatography and resuspended in DMSO. The synthetic autoinducing peptide was confirmed to match the spent medium of *S. hominis* C5 autoinducing peptide structure by running 0.05 mg/ml of the synthetic peptide side by side with the spent medium and measuring equivalent fragmentation patterns, retention times, and mass.

### TEWL measurements

To determine damage to the epidermal skin barrier, TEWL of murine skin treated for 48 to 72 hours with *S. aureus* was measured using a Tewameter TM300 (C & K).

### Trypsin activity analysis

NHEK conditioned medium was added at 50  $\mu\text{l}$  to 96-well black-bottom plates (Corning) followed by addition of 150  $\mu\text{l}$  of the peptide Boc- Val-Pro-Arg-AMC (trypsin-like substrate;

Bachem) at a final concentration of 200  $\mu$ M in 1 $\times$  digestion buffer [10 mM tris-HCl (pH7.8)] and incubated at 37°C for 24 hours. Relative fluorescence intensity (excitation, 354 nm; emission, 435 nm) was analyzed with a SpectraMax Gemini EM fluorometer (Thermo Fisher Scientific). For murine skin trypsin activity analysis, 0.5-cm<sup>2</sup> full-thickness skin was bead-beat (2.0-mm zirconia beads, 2  $\times$  30 s with 5 min after each) in 1 ml of 1 M acetic acid, followed by an overnight rotation at 4°C. Samples were centrifuged (10 min, 13,000 rpm, 4°C) and then added to a new microcentrifuge tube followed by protein concentration using a SpeedVac to remove all remaining acetic acid. Proteins were resuspended in molecular-grade water (500  $\mu$ l) and rotated overnight at 4°C followed by another centrifugation. Clear protein lysates were added to a new tube, and bicinchoninic acid (Thermo Fisher Scientific) analysis was used to determine protein concentration. Last, 10  $\mu$ g of total protein was added to a 96-well plate followed by analysis with the trypsin substrate as above.

### ***S. aureus agr* activity**

Either the *S. aureus* USA300 LAC *agr* type I P3-YFP (AH1677) or the *S. aureus* USA300 LAC *agr* type I pAmi P3-Lux (AH2759) reporter strains were used to detect *S. aureus agr* activity. For in vitro experiments, *S. aureus* USA300 LAC *agr* type I P3-YFP diluted to 1  $\times$  10<sup>6</sup> CFU and added to 300  $\mu$ l of 3% TSB along with 100  $\mu$ l of sterile-filtered commensal supernatant (25% by volume) was cultured overnight and shaken (300 rpm) for 24 hours at 37°C. Bacteria were then diluted 1:20 in phosphate-buffered saline (PBS) (200  $\mu$ l final), and yellow fluorescent protein (YFP) (excitation, 495 nm; emission, 530 nm) was detected using the fluorometer as above. Bacterial density was determined using an OD<sub>600nm</sub> readout on a spectrophotometer. For murine experiments, *S. aureus* USA300 LAC *agr* type I pAmi P3-Lux luminescence activity was determined using a Spectrum IVIS machine. Luminescence intensity was assessed after an automatic 2-min exposure by measuring emitted photons (p/s/cm<sup>2</sup>/sr) using the Live Imaging software (PerkinElmer).

### **Microbiome data and comparative genomic analysis**

We analyzed publicly available shotgun metagenomic data for AD skin (28). The relative abundance of *S. aureus* and *S. epidermidis* strains was obtained directly from the published supplementary material ([www.sciencetranslationalmedicine.org/cgi/content/full/9/397/eaal4651/DC1](http://www.sciencetranslationalmedicine.org/cgi/content/full/9/397/eaal4651/DC1)). The *agrD* characterization analysis was performed on all 11 available AD patients with information at seven distinct body sites on flared AD skin (AD01 to AD11), with differences in AD severity based on oSCORAD. Both *S. aureus* and *S. epidermidis* strains detected from this dataset were classified by *agr* type through amino acid sequence comparison with known *agr* sequences within the *agrD* gene region (43).

### **Quantification and statistical analysis**

The (nonparametric) unpaired Kruskal-Wallis test was used for statistical significance analysis of AD patient metagenomic data in Fig. 2G and AD skin swabs analysis of *psma* expression in Fig. 3H and fig. S4. All other figures used Student's *t* tests and one-way analysis of variance (ANOVA) for statistical analysis, as indicated in the figure legends. All statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad). All data were presented as means  $\pm$  SEM, and a *P* value of  $\leq$  0.05 was considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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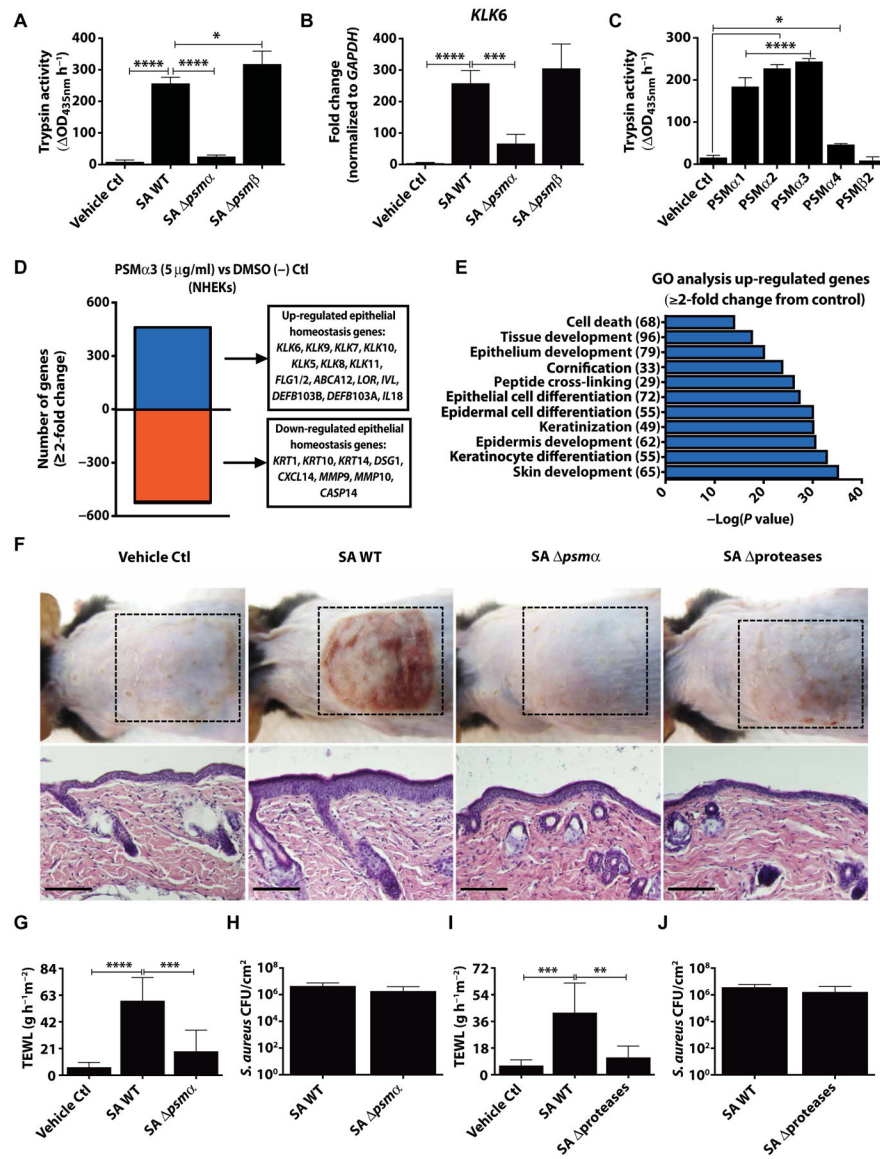
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**Fig. 1. *S. aureus* PSM $\alpha$  induces keratinocyte protease activity and disrupts epithelial barrier homeostasis.**

NHEKs were stimulated with 5% *S. aureus* USA300 LAC (SA) supernatant from overnight cultures [ $1 \times 10^9$  colony-forming units (CFU)] of wild-type (WT) and *psma* (*psma*) or *psmb* (*psmb*) knockout strains for 24 hours. Both (A) trypsin activity and (B) *KLK6* mRNA expression compared to the housekeeping gene *GAPDH* were analyzed ( $n = 4$ ). (C) PSM synthetic peptides were added to NHEKs for 24 hours to analyze changes in trypsin activity ( $n = 4$ ). (D) Transcript analysis by RNA-seq and (E) gene ontology (GO) analysis of genes that changed  $\geq 2$ -fold 24 hours after PSM $\alpha$ 3 treatment. The number of genes in each category shown in parentheses. (F) Eight-week-old male C57BL/6 mice were treated for 72 hours with  $1 \times 10^7$  CFU SA WT (USA300 LAC) and corresponding SA *psma*, or SA USA300 LAC WT (AH1263) and corresponding SA  $\Delta proteases$  [SA WT in (F) representative of both SA WT strains]. Bacterial growth medium was used as a vehicle control. Dashed lines indicate treatment area. Scale bars, 200  $\mu$ m ( $n = 6$ ). (G to J)

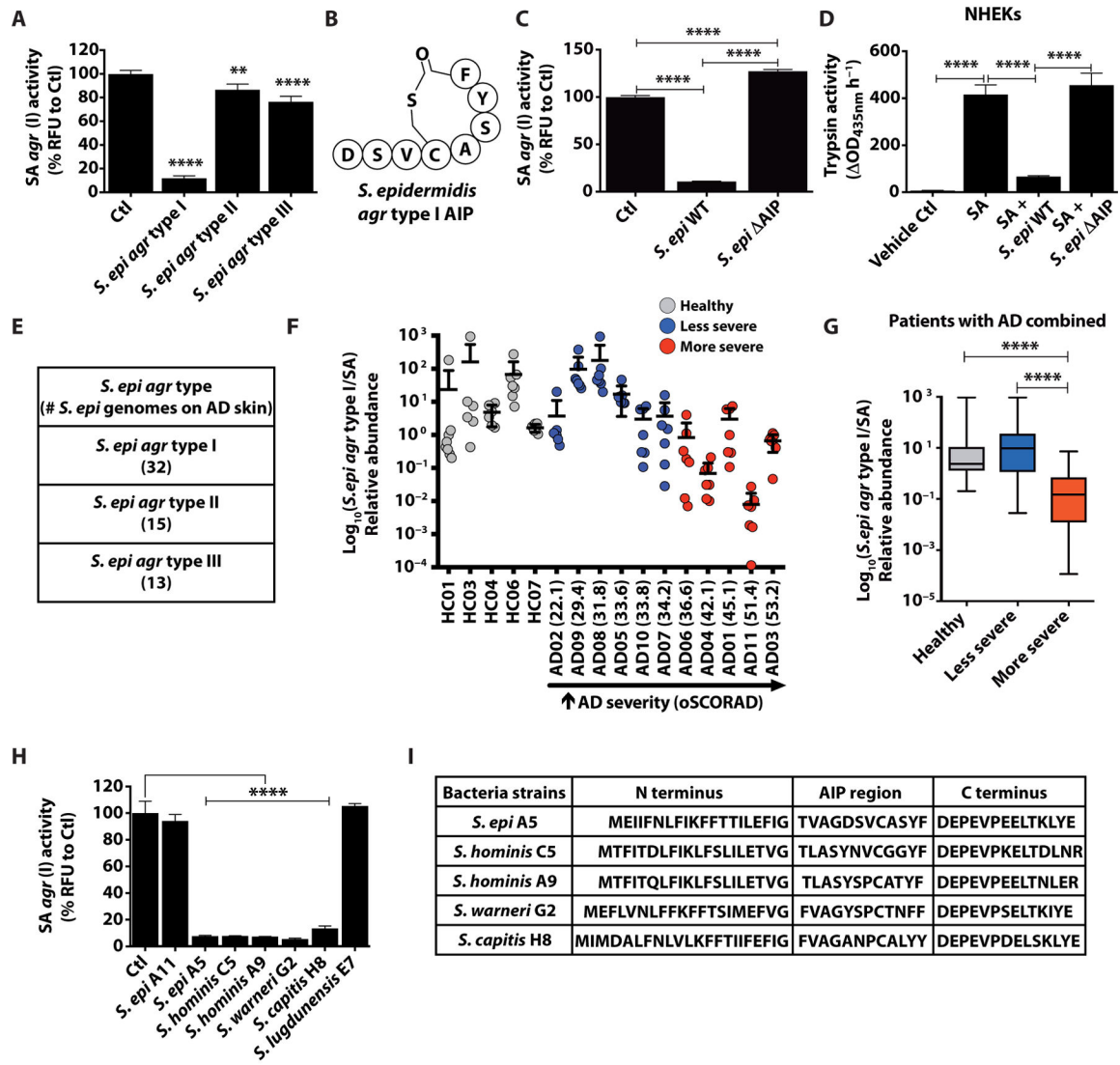
Transepidermal water loss (TEWL) or measurement of CFU/cm<sup>2</sup> on mice after 72-hour treatment with initial  $1 \times 10^7$  CFU of (G and H) SA WT (USA300 LAC) and corresponding SA *psma* or (I and J) SA WT [USA300 LAC (AH1263)] and corresponding SA proteases ( $n = 6$ ). All experiments are representative of three independent experiments. Error bars are SEM. One-way ANOVA was used to determine statistical significance. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . Ctl, control.

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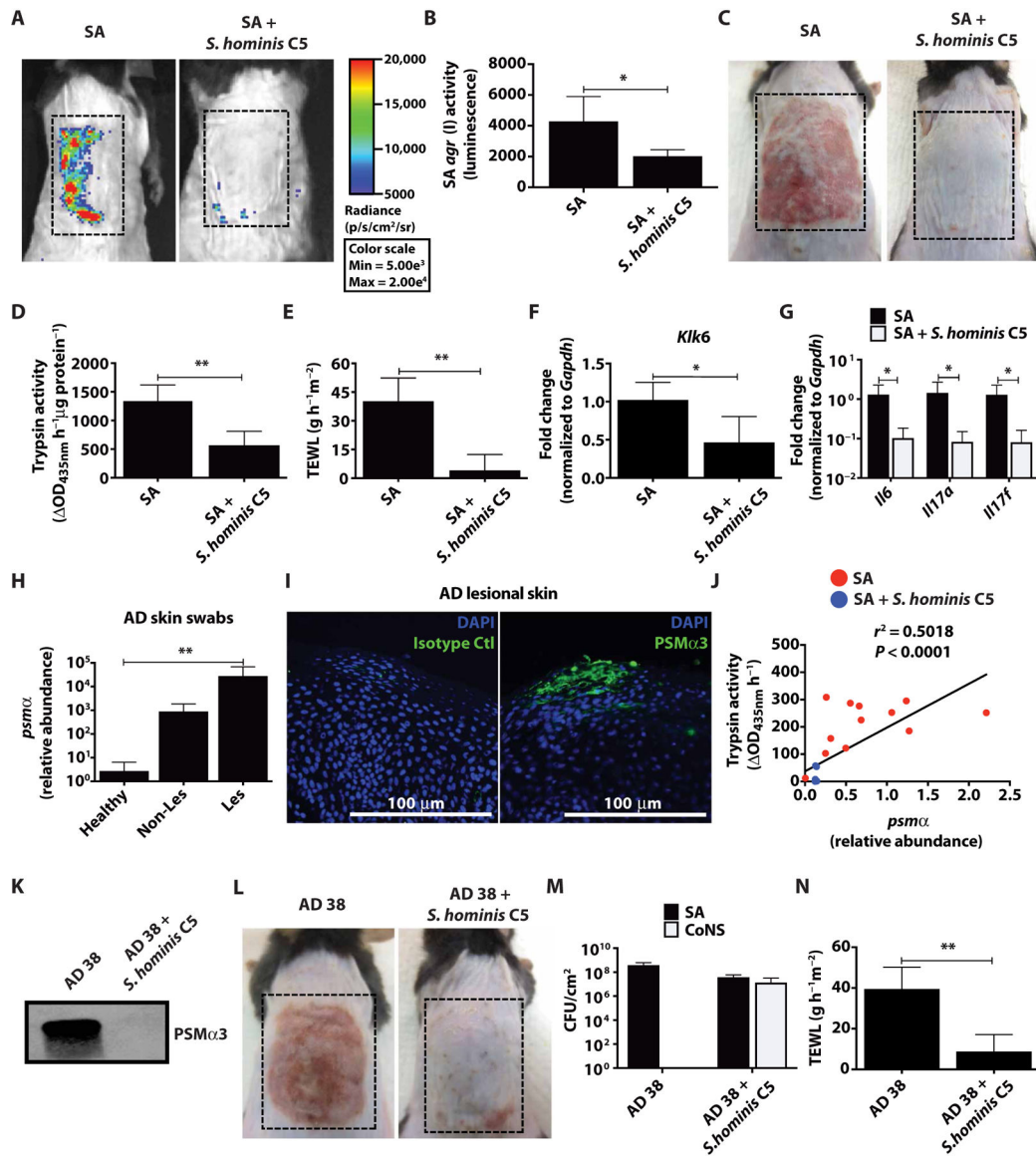
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**Fig. 2. *S. epidermidis* agr type I autoinducing peptide characterization and deficiency in AD skin.** (A) SA USA300 LAC *agr* type I P3-YFP activity after overnight culture ( $1 \times 10^9$  CFU) with 25% supernatant from overnight culture of *S. epidermidis* (*S. epi*) *agr* types I (RP62A), II (1457), and III (8247) ( $n = 4$ ). (B) Structure of *S. epidermidis* *agr* type I autoinducing peptide (AIP). (C) Analysis of *agr* activity as in (A) with *S. epidermidis* *agr* type I (RP62A) WT or autoinducing peptide knockout ( $\Delta$ AIP) strain supernatants ( $n = 4$ ). (D) NHEK trypsin activity after culture for 24 hours with 5% SA supernatant grown overnight with or without *S. epidermidis* *agr* type I (RP62A) WT or autoinducing peptide knockout ( $\Delta$ AIP) ( $n = 4$ ). (E) Number (#) of *S. epidermidis* *agr* types I to III strains found on AD skin from metagenomic dataset. (F and G) Ratio of *S. epidermidis* *agr* type I to SA relative abundance on the combined analysis of all sites sampled from 5 healthy individuals and 11 AD subjects during flare and ranked from “least severe” to “most severe” based on oSCORAD. (H) Analysis of *agr* activity as in (A) after coculture overnight with 25% supernatant of overnight cultures ( $1 \times 10^9$  CFU) of clinical CoNS strains ( $n = 3$ ). (I) Assessment of autoinducing peptide coding regions in *agrD* gene of the inhibitory CoNS strains. All experiments are representative of

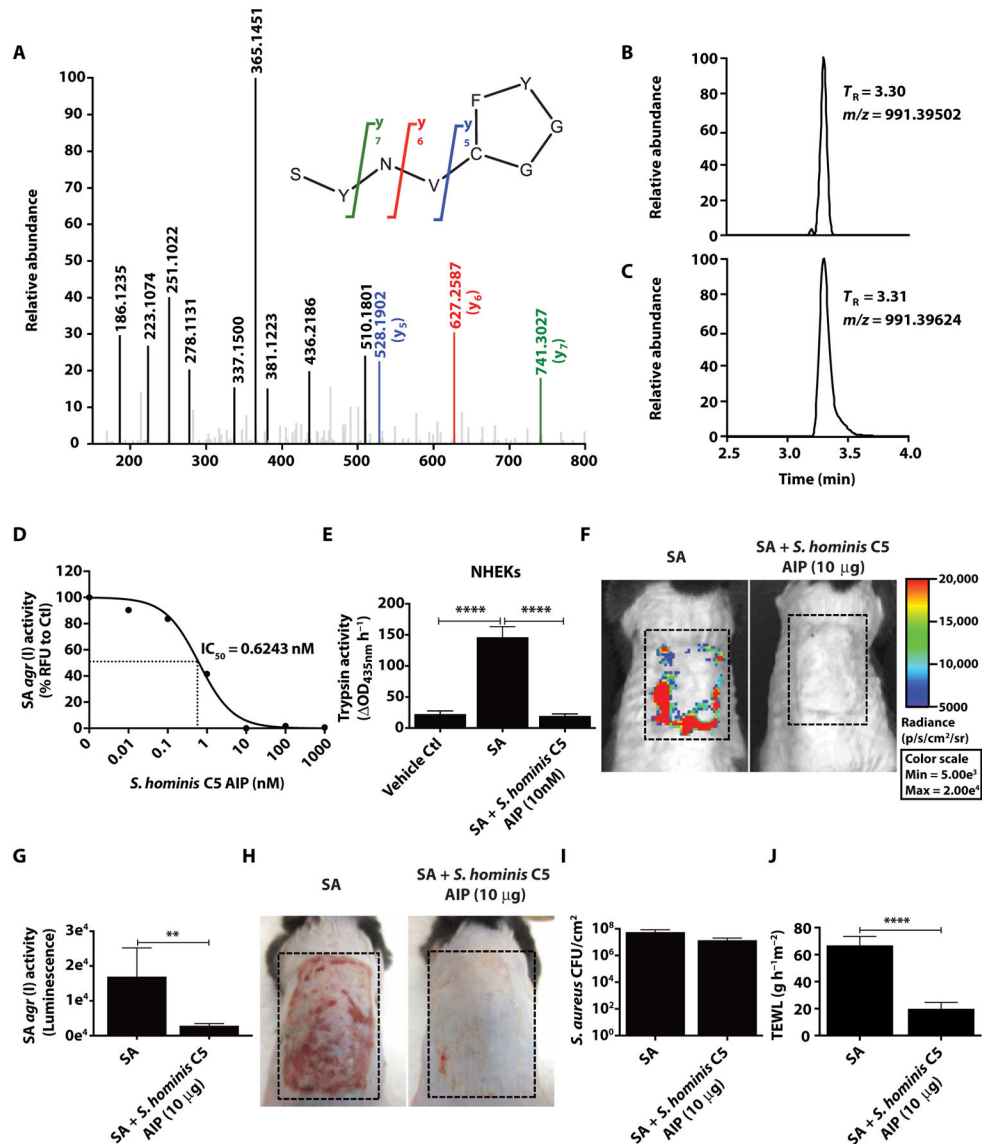
three independent experiments. All error bars are SEM. One-way ANOVA or an (nonparametric) unpaired Kruskal-Wallis test in (G) was used to determine statistical significance. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .



**Fig. 3. Inhibition of *S. aureus agr* activity by clinical *S. hominis* isolate correlates with prevention of skin barrier damage and inflammation.**

(A to C) SA USA300 LAC *agr* type I pAmi P3-Lux reporter strain (AH2759) ( $1 \times 10^7$  CFU) combined with or without live *S. hominis* C5 ( $1 \times 10^7$  CFU) was applied to murine back skin for 72 hours, and SA *agr* activity was assessed by changes in luminescence. Images are representative of  $n = 5$ ; dashed boxes indicate treatment area. (D to G) Murine back skin after 72-hour bacteria treatment, assessed for changes in TEWL, trypsin activity, and *Klf6* and cytokine (*Il6*, *Il17a*, and *Il17f*) mRNA expression normalized to housekeeping gene *Gapdh* ( $n = 5$ ). (H) Relative abundance of SA *psmα* mRNA isolated from swabs of healthy ( $n = 6$ ) and AD nonlesional and lesional skin ( $n = 5$ ). (I) Detection of SA PSM $\alpha$ 3 by immunofluorescence in epidermis of AD lesional skin (representative image with scale bar, 100  $\mu$ m). (J) Clinical SA isolates from 11 patients were grown up to 18 hours ( $1 \times 10^9$  CFU) with or without 25% *S. hominis* C5 supernatant. *psmα* mRNA expression was measured at 8 hours, whereas trypsin activity was assessed from NHEKs treated 24 hours with 18-hour

cultured SA (5%) supernatant ( $n = 3$ ). **(K)** Immunoblot of culture supernatant from clinical SA isolate AD 38 grown overnight ( $1 \times 10^9$  CFU) with or without *S. hominis* C5 supernatant and probed for PSM $\alpha$ 3. **(L to N)** SA AD 38 ( $1 \times 10^7$  CFU) was applied to murine back skin with or without *S. hominis* C5 ( $1 \times 10^7$  CFU) for 72 hours ( $n = 5$ ) (dashed boxes indicate treatment area). Changes in CFU/cm<sup>2</sup> of SA and CoNS, and TEWL are shown. All experiments are representative of two independent experiments. Error bars represent SEM. Student's *t* tests, Pearson correlation coefficient (J), and an (nonparametric) unpaired Kruskal-Wallis test (H) were used to determine statistical significance. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .



**Fig. 4. Identification of an autoinducing peptide from a clinical *S. hominis* isolate that inhibits *S. aureus agr* activity.**

(A) Determination of *S. hominis* C5 autoinducing peptide sequence as SYNVCGGYF. The MS-MS spectrum obtained with the synthetic peptide is shown. Bolded fragment peaks and those indicated in color were identified in the MS-MS spectra for both the synthetic peptide and the spent growth medium sample (with difference in mass of <5 parts per million). Only matching fragments above 15% abundance are shown. Key features used for identification were the  $y_5$  (blue),  $y_6$  (red), and  $y_7$  (green) fragments of the cyclic autoinducing peptide. The selected-ion chromatograms for mass/charge ratio ( $m/z$ )  $991.3984 \pm 5$  ppm for (B) bacteria culture and (C) synthetic autoinducing peptide in growth medium confirmed the retention time ( $T_R$ ) and accurate  $m/z$  of the predicted autoinducing peptide sequence. (D) Analysis of *S. hominis* C5 synthetic autoinducing peptide on SA USA300 LAC *agr* type I P3-YFP activity after an overnight culture ( $1 \times 10^9$  CFU) and  $IC_{50}$  value indicated by dotted lines at midpoint of the curve. (E) Measurement of NHEK trypsin activity after 24-hour incubation



with 5% supernatant from overnight culture of SA with or without *S. hominis* C5 autoinducing peptide ( $1 \times 10^9$  CFU) (*F* to *H*) SA USA300 LAC *agr* type I pAmi P3-Lux with or without *S. hominis* C5 synthetic autoinducing peptide was colonized ( $1 \times 10^7$  CFU) on murine back skin for 48 hours followed by analysis of *agr* activity (luminescence) and representative pictures of murine back skin after treatment (dashed lines represent treated areas) (*n* = 5). (*I* and *J*) SA CFU/cm<sup>2</sup> and TEWL after treatment with *S. hominis* C5 synthetic autoinducing peptide. Data are representative of two experiments. Error bars are SEM. One-way ANOVA and Student's *t* tests were used to determine statistical significance. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.