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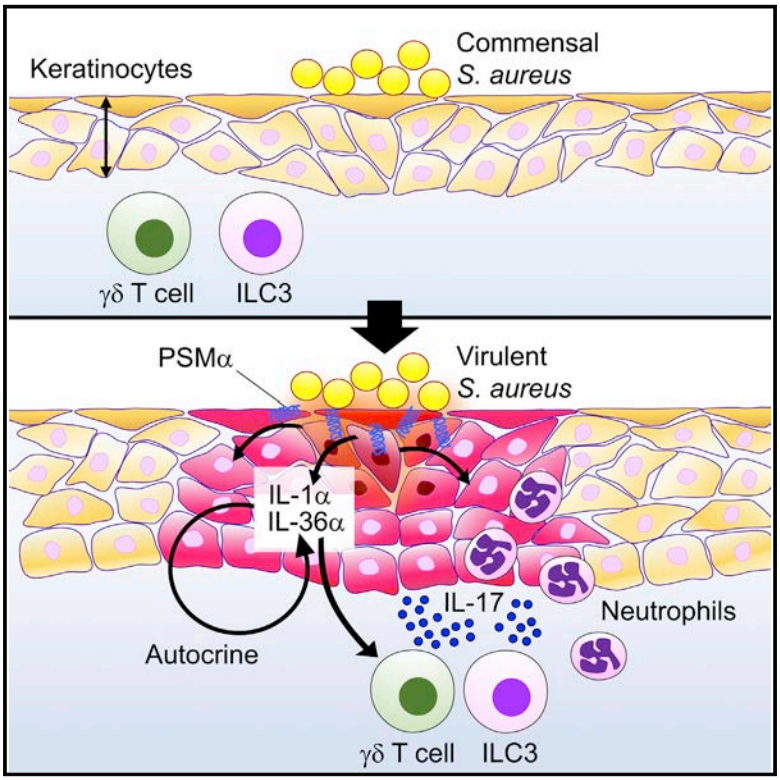
Staphylococcus aureus Virulent PSMα Peptides Induce Keratinocyte Alarmin Release to Orchestrate IL-17-Dependent Skin Inflammation

Graphical Abstract



Highlights

- *S. aureus* virulence factor PSMα induces the release of keratinocyte IL-1α and IL-36α
- Myd88 signaling in keratinocytes is required for IL-1α and IL-36α production
- IL-1R and IL-36R signaling is critical for the induction of IL-17-producing cells
- Mice deficient in IL-17A/F show blunted *S. aureus*-induced skin inflammation

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In Brief

Nakagawa et al. demonstrate that *S. aureus* produces PSMα, a group of virulence peptides, to induce keratinocyte damage and release of IL-1α and IL-36α. IL-1R and IL-36R signaling via the adaptor Myd88 induces IL-17-producing γδ T cells and ILC3, which critically mediate skin inflammation in response to epicutaneous *S. aureus.*

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Staphylococcus aureus Virulent PSMα Peptides Induce Keratinocyte Alarmin Release to Orchestrate IL-17-Dependent Skin Inflammation

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SUMMARY

Staphylococcus aureus **commonly colonizes the epidermis, but the mechanisms by which the host senses virulent, but not commensal, *S. aureus* to trigger inflammation remain unclear. Using a murine epicutaneous infection model, we found that *S. aureus*-expressed phenol-soluble modulin (PSM)α, a group of secreted virulence peptides, is required to trigger cutaneous inflammation. PSMα induces the release of keratinocyte IL-1α and IL-36α, and signaling via IL-1R and IL-36R was required for induction of the pro-inflammatory cytokine IL-17. The levels of released IL-1α and IL-36α, as well as IL-17 production by γδ T cells and ILC3 and neutrophil infiltration to the site of infection, were greatly reduced in mice with total or keratinocyte-specific deletion of the IL-1R and IL-36R signaling adaptor Myd88. Further, *Il17a*$^{-/-}$*f*$^{-/-}$ mice showed blunted *S. aureus*-induced inflammation. Thus, keratinocyte Myd88 signaling in response to *S. aureus* PSMα drives an IL-17-mediated skin inflammatory response to epicutaneous *S. aureus* infection.**

INTRODUCTION

The skin is the largest organ at the interface between the external environment and host tissues. The epidermis located on the skin surface is important in maintaining the physical and immunological barrier of the skin by protecting the host from harmful environmental stimuli, including invasive microbes (Segre, 2006). *Staphylococcus aureus*, a Gram-positive bacterium, is a leading cause of human infection capable of invading most tissues of the human body. The superficial skin is a major infection site for *S. aureus*, which normally resides in 10%–20% of healthy individuals (Lowy, 1998). The *S. aureus*-causing skin infections often originate from resident bacteria that colonize the mucosal surfaces of the skin (Balasubramanian et al., 2017; Lowy, 1998). How *S. aureus* produces virulence factors to transform from a skin commensal to a pathogen is poorly understood. Previous studies suggested that this might be a consequence of activation of virulence gene regulatory networks in response to environmental signals (Novick and Geisinger, 2008). A major *S. aureus* virulence program is the accessory gene regulatory (Agr) quorum sensing, a two-component system that responds to bacterial density (Novick, 2003). Upon activation, the *agr* locus induces the expression of a wide array of secreted virulence factors, including toxins and enzymes that are important for the adaptation of the pathogen to the environment (Novick, 2003). Agr-regulated toxins include phenol-soluble modulins (PSMs), a group of seven different peptides divided into α- and β-type PSMs (Cheung et al., 2014). PSM peptides form amphipathic α-helical structures capable of forming pores in artificial membranes (Wang et al., 2007). PSMα peptides are highly cytotoxic to a wide variety of cells including keratinocytes (KCs), while other PSMs have very limited cytotoxic activities (Nakamura et al., 2013; Wang et al., 2007). In a mouse epicutaneous model of *S. aureus* infection, δ-toxin, a PSM peptide, promotes skin inflammation by inducing mast cell degranulation (Nakamura et al., 2013). However, the mechanism by which *S. aureus* interacts with KCs to trigger skin inflammation *in vivo* remains largely unknown.

KCs are the predominant cell type in the stratified epithelial layer of the epidermis. In response to environmental stimuli, KCs produce a wide variety of molecules including multiple cytokines, chemokines, and anti-microbial peptides (Kennedy-Crispin et al., 2012; Nestle et al., 2009). Some of these KC molecules, including IL-1α, high-mobility group box 1 (HMGB1) protein, and anti-microbial peptides, can be released upon tissue damage and function as "alarmins" to activate the immune system (Rider et al., 2017; Yang et al., 2009). However, the role of KCs in *S. aureus*-induced inflammation *in vivo* is poorly understood because practically all cutaneous models of *S. aureus* infection rely on subepidermal inoculation or prior physical disruption of the epidermis (Miller et al., 2006; Wang et al., 2007). In the intradermal or subcutaneous model of

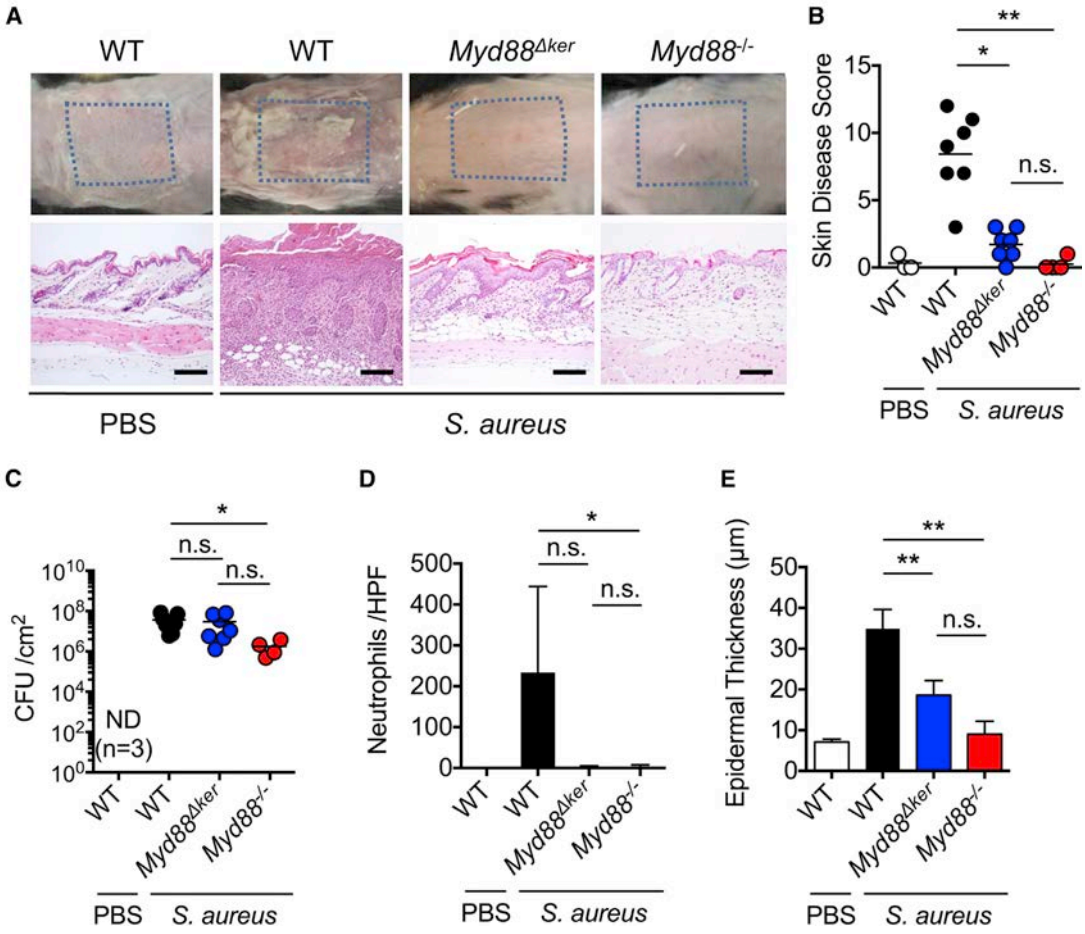

Figure 1. Keratinocyte Myd88 Is Important for Epicutaneous *S. aureus*-Induced Skin Inflammation

(A) C57BL/6 (WT), *K14-CreMyd88*$^{fl/fl}$ (*Myd88*$^{\Delta ker}$), and *Myd88*$^{-/-}$ mice were epicutaneously colonized with *S. aureus*. WT mice treated with PBS are shown for comparison. Representative macroscopic images and hematoxylin and eosin (HE)-stained skin sections 7 days after colonization (n = 3–7 mice per group). Scale bars, 100 μm.

(B) Day 7 skin disease scores of WT, *Myd88*$^{\Delta ker}$, and *Myd88*$^{-/-}$ mice colonized with *S. aureus*.

(C–E) The number of *S. aureus* colony-forming units (CFUs) (C), numbers of neutrophils per high power field (D), and epidermal thickness (E) in the skin of WT, *Myd88*$^{\Delta ker}$, and *Myd88*$^{-/-}$ mice 7 days after colonization with *S. aureus*.

Each dot represents a mouse (B and C). Data are presented as mean ± SD (D and E). Results shown represent combined data of two independent experiments. ND, not detected; n.s., not significant; *p < 0.05, **p < 0.01, by Kruskal-Wallis test.

S. aureus infection, mice deficient in Myd88, the adaptor molecule that is critical for signaling through the Toll-like receptor (TLR)/IL-1/IL-18 family of receptors, showed increased inflammation, abscess formation, and epidermal ulceration that correlated with impaired *S. aureus* clearance (Miller et al., 2006). Furthermore, *Il1r*$^{-/-}$, but not *Tlr2*$^{-/-}$, mice showed increased inflammation and abscess formation in the subcutaneous infection model (Miller et al., 2006). However, the role of these signaling pathways in promoting inflammation after epidermal colonization of *S. aureus* remains unclear. Using conditions in which virulence genes are induced upon epidermal colonization, we show that *S. aureus* relies on virulent Agr-regulated PSMα peptides to trigger cutaneous inflammation. PSMα induces the release of IL-1α and IL-36 from KCs to orchestrate cutaneous inflammation via Myd88 signaling and IL-17 production.

RESULTS

Keratinocyte Myd88 Is Essential for *S. aureus*-Induced Skin Inflammation

We used a recently developed model of *S. aureus* colonization that induces Agr-regulated virulence to investigate the mechanism by which the pathogen triggers cutaneous inflammation (Nakamura et al., 2013). In this epicutaneous model, wild-type (WT) mice infected with *S. aureus* (strain LAC, pulsed-field type USA300) showed severe skin disease and inflammation characterized by neutrophil infiltrates and epidermal thickening on day 7 after colonization (Figures 1A, 1B, 1D, and 1E). In contrast, *Myd88*$^{-/-}$ mice showed little skin pathology when compared with WT mice (Figures 1A–1E). Unlike the epicutaneous model, *Myd88*$^{-/-}$ mice infected intradermally with *S. aureus* showed increased skin pathology and pathogen loads compared to

that observed in WT-infected mice (Figures S1A–S1C), which is consistent with a previous report (Miller et al., 2006). In contrast, *Myd88*$^{-/-}$ mice exhibited lower *S. aureus* colony-forming units (CFUs) than WT mice in the epicutaneous model (Figure 1C), suggesting that Myd88-dependent inflammation promotes pathogen colonization in this model. Thus, the route of infection is critical to revealing the function of innate signaling pathways in host defense against *S. aureus* in the skin. To determine whether Myd88 acts within KCs, we crossed mice with a floxed exon of Myd88 (*Myd88*$^{fl/fl}$ mice) with a keratin 14 (K14)-Cre mouse deleter strain to generate *K14-Cre-Myd88*$^{fl/fl}$ mice with conditional deletion of Myd88 within KCs. Notably, mice lacking Myd88 in KCs (*Myd88*$^{\Delta ker}$) showed a phenotype similar to that observed in mice with whole-body deletion of Myd88 (Figures 1A, 1B, and 1D). However, pathogen loads were comparable in *Myd88*$^{\Delta ker}$ mice and WT mice (Figure 1C). Furthermore, the reduction in epidermal thickening in *Myd88*$^{\Delta ker}$ mice was not as prominent as in *Myd88*$^{-/-}$ mice (Figure 1E). In contrast to the epicutaneous model, *Myd88*$^{\Delta ker}$ and WT mice showed comparable skin inflammation in response to intradermal *S. aureus* infection (Figures S1D–S1F). These results indicate a critical role for Myd88 and particularly Myd88 in KCs for the induction of immune responses to *S. aureus* in the epicutaneous infection model.

IL-1R and IL-36R Are Essential for Induction of Skin Inflammation in Epicutaneous *S. aureus* Infection

Myd88 is a critical adaptor molecule required for signaling via multiple immune receptors, including TLR/IL-1R/IL-18R/IL-36R (Palomo et al., 2015; Takeuchi and Akira, 2002). Initial experiments showed that deficiency in TLR2, TLR4, or IL-18 did not affect skin disease, histopathology, or pathogen loads in the epicutaneous model of *S. aureus* infection (Figures S2A and S2B). In contrast, mice deficient in IL-1R, which can be stimulated by either IL-1α or IL-1β, exhibited a reduction in disease score, neutrophil infiltrates, and epidermal thickening, despite similar pathogen loads when compared with WT mice (Figures 2A–2E). Two related cytokines, IL-36α and IL-36γ, are expressed in KCs (Gresnigt and van de Veerdonk, 2013) and activate the IL-36R, which also signals via Myd88 (Towne et al., 2004). Administration of a neutralizing monoclonal antibody (Mab) against IL-36R also reduced neutrophil infiltration and epidermal thickening induced by epicutaneous *S. aureus* infection without affecting pathogen loads (Figures 2C–2E). Treatment of *Il1r*$^{-/-}$ mice with the neutralizing anti-IL-36R Mab further reduced the disease score, neutrophil infiltration, and epidermal thickening when compared with WT mice (Figures 2A, 2B, 2D, and 2E). These results indicate that both IL-1R and IL-36R signaling contribute to skin inflammation induced by *S. aureus* infection.

S. aureus Induces IL-1α and IL-36α Expression via Myd88 in Keratinocytes

Given that IL-1R signaling is important for *S. aureus*-induced inflammation, we asked whether IL-1α or IL-1β mediates skin pathology in the epicutaneous infection model. Infection with *S. aureus* showed comparable pathology, disease scores, and pathogen loads in WT and *Il1β*$^{-/-}$ mice (Figure S2C). In contrast, neutralization of IL-1α with a Mab reduced skin pathology without affecting pathogen loads, compared with mice treated with isotype-matched control Mab (Figures 3A–3C). Epicutaneous infection of *S. aureus* in WT mice substantially enhanced the expression of IL-1α and IL-36α in the upper area of the epidermis, which was reduced in *Myd88*$^{\Delta ker}$ and *Myd88*$^{-/-}$ mice (Figures 3D and S3). Consistent with results showed in Figure 1C, pathogen loads were reduced in the skin of *Myd88*$^{-/-}$ mice compared to WT and *Myd88*$^{\Delta ker}$ (Figure 3E). To determine whether *S. aureus* produces molecules that induce IL-1α and IL-36α release from KCs, primary KCs from WT and *Myd88*$^{-/-}$ mice were cultured under conditions that promote their terminal differentiation (Bikle et al., 2012) and then incubated with supernatants of *S. aureus* cultures. There was marked release of IL-1α and IL-36α after 60 min incubation of WT KCs with *S. aureus* culture supernatants (Figures 3F and 3G). Notably, the release of IL-1α and IL-36α was reduced in KCs from *Myd88*$^{-/-}$ mice (Figures 3F and 3G). Furthermore, the KC release of IL-1α was markedly reduced and that of IL-36α was partially affected in KCs deficient in IL-1R (Figures 3H and 3I), suggesting that IL-1α release enhances IL-1α and IL-36α production via IL-1R signaling in KCs. In addition, IL-36α release by *S. aureus* was inhibited by neutralization of IL-36R, whereas that of IL-1α was only minimally affected (Figures 3J and 3K). Collectively, these results indicate that IL-1α and IL36α production is regulated by *S. aureus* stimulation via Myd88 signaling in KCs.

IL-17 Is Critical for *S. aureus*-Induced Skin Inflammation

Members of the IL-1 family of cytokines are important for the induction of cellular immune responses and production of several cytokines, including IL-17 and IL-22 (Sonnenberg et al., 2011; Villarino and Laurence, 2015). To determine which cytokines are induced upon epicutaneous infection with *S. aureus*, we assessed the production of multiple cytokines by skin immune cells at the peak of inflammation. Total skin cells were gated on hematopoietic CD45^{+}CD90^{+} cells and the number of CD45^{+}CD90^{+} cells producing IL-17A, IL-17F, IL-22, interferon-γ (IFN-γ), and GM-CSF was measured by flow cytometry. The analysis revealed that epicutaneous *S. aureus* infection induces a marked increase in the number of IL-17A-producing cells and a modest increase in cells producing IL-17F and IL-22 while there was minimal or no induction of IFN-γ- and GM-CSF-producing cells (Figure 4A). The number of leukocytes infiltrating the skin, including IL-17A-producing cells, was reduced in *Myd88*$^{-/-}$ mice (Figure 4B). Furthermore, the production of IL-17A was reduced in the skin of *Myd88*$^{\Delta ker}$ and *Il1r*$^{-/-}$ mice injected with IL-36R-neutralizing Mab (Figure 4C). The production of IL-17F was also reduced in *Il1r*$^{-/-}$ mice injected with IL-36R-neutralizing Mab compared to WT mice (Figure 4C). To determine if IL-17 was important for the induction of skin inflammation, WT mice and mice doubly deficient in IL-17A and IL-17F were infected epicutaneously with *S. aureus*. We found that *Il17a*$^{-/-}$*f*$^{-/-}$ mice exhibited greatly reduced skin disease scores and neutrophil infiltration, but normal pathogen loads, compared with WT mice (Figures 4D–4H). In contrast, WT and *Il22*$^{-/-}$ mice showed comparable skin phenotype after *S. aureus* infection (Figure S2D). These results indicate that *S. aureus* induces IL-17 via Myd88 signaling, which is critical for inflammatory pathology in the skin.

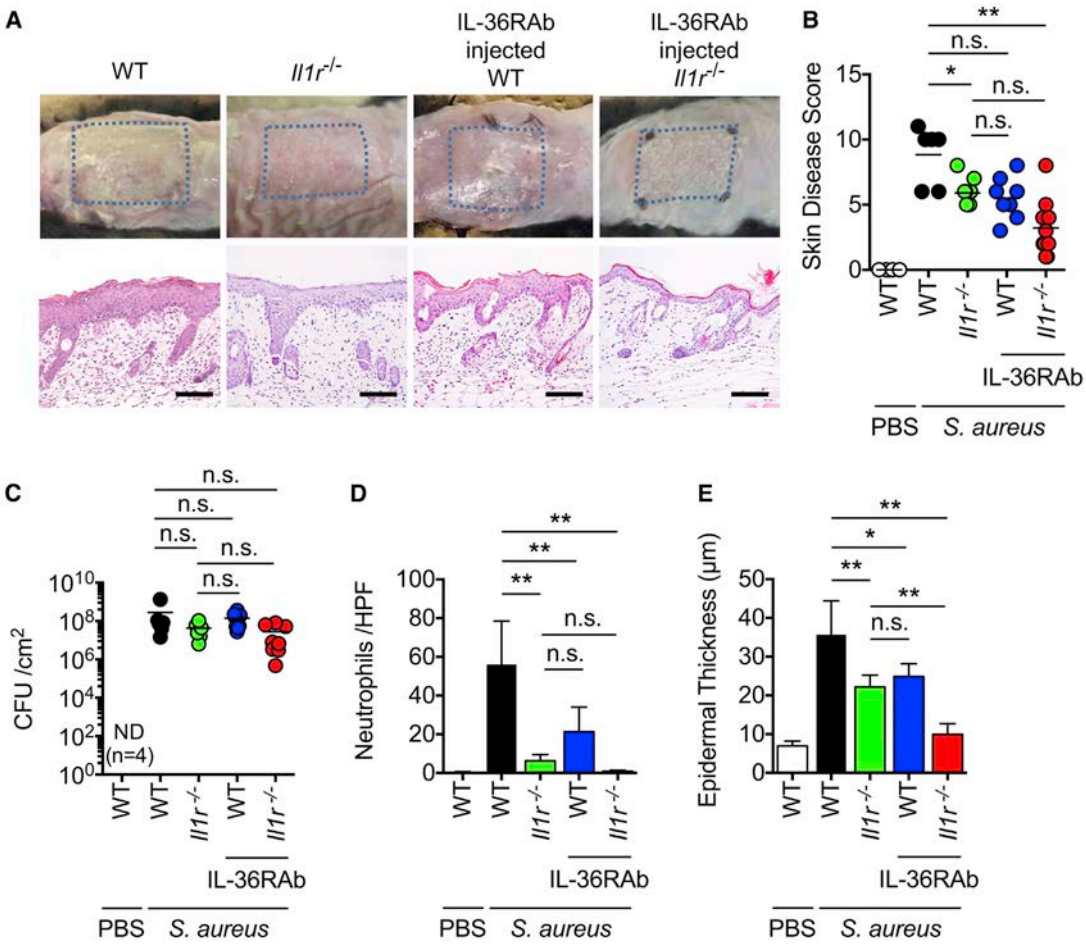

Figure 2. Both IL-1R and IL-36R Contribute to Skin Inflammation Induced by Epicutaneous *S. aureus* Colonization

(A) WT, *Il1r*$^{-/-}$, IL-36R antibody (IL-36RAb)-treated WT, and IL-36RAb-treated *Il1r*$^{-/-}$ mice were epicutaneously colonized with *S. aureus* for 7 days. Representative macroscopic images and HE-stained skin sections of mice colonized with *S. aureus* or treated with PBS (n = 4–8 mice per group). Scale bars, 100 μm. (B–E) Day 7 skin disease scores (B), *S. aureus* CFUs in the skin (C), the number of neutrophils in the skin (D), and epidermal thickness (E) of WT, *Il1r*$^{-/-}$, IL-36RAb-treated WT, and IL-36RAb-treated *Il1r*$^{-/-}$ mice (n = 4–8 mice per group). WT mice treated with PBS are shown for comparison.

Each dot represents a mouse (B and C). Data are presented as mean ± SD (D and E). Data represent combined results from three independent experiments. ND, not detected; n.s., not significant; *p < 0.05, **p < 0.01, by Kruskal-Wallis test (B and C) or by one-way ANOVA test with Bonferroni's correction (D and E).

γδ T Cells and Type 3 Innate Lymphoid Cells Are the Main Producers of IL-17 in Response to Skin Infection

We next asked which immune cell population(s) produces IL-17 in response to epicutaneous *S. aureus* infection. Gating on CD45$^+$ cells revealed two populations of IL-17$^+$CD90$^+$ cells in the skin of mice epicutaneously infected with *S. aureus* (Figure 5A). These included γδ^{intermediate} dermal γδ T cells and lineage-negative CD45$^+$CD90$^+$ immune cells, which mark type 3 innate lymphoid cells (ILC3s) (Figure 5A). To determine whether γδ T cells contribute to skin inflammation in response to *S. aureus*, we infected WT and *Tcrδ*$^{-/-}$ mice with the pathogen and assessed the pathology and disease scores in the skin. We found comparable disease scores, but reduced neutrophil infiltration, in *Tcrδ*$^{-/-}$ mice compared with WT mice (Figures 5B–5F). As expected, IL-17-producing γδ T cells were absent in the skin of infected *Tcrδ*$^{-/-}$ mice, but the population of IL-17-producing ILCs was unchanged in *Tcrδ*$^{-/-}$ mice (Figure 5G). To determine whether ILC3s contribute to skin inflammation, we treated *Tcrδ*$^{-/-}$ mice with anti-CD90 Mab to deplete the ILC3 population (Figure S4) and infected the treated mice epicutaneously with *S. aureus*. Treatment with anti-CD90 Mab significantly reduced skin disease scores without affecting pathogen loads in *Tcrδ*$^{-/-}$ mice when compared with mutant mice treated with control Mab (Figures 5H–5J). These results indicate that both ILC3s and γδ T cells contribute to skin inflammation in response to epicutaneous *S. aureus* infection.

S. aureus PSMα Induces Keratinocyte IL-1α and IL-36α Release to Mediate Skin Inflammation

We next assessed which factor(s) released by *S. aureus* is important for eliciting the release of IL-1α and IL-36α from KCs and thereby triggering IL-17-dependent skin inflammation. PSMs, including the peptides PSMα1 through PSMα4, induce robust cell death of mouse KCs *in vitro* (Nakamura et al., 2013). Because the Agr virulence system that controls the production of PSMα is activated during epicutaneous *S. aureus* infection in our model

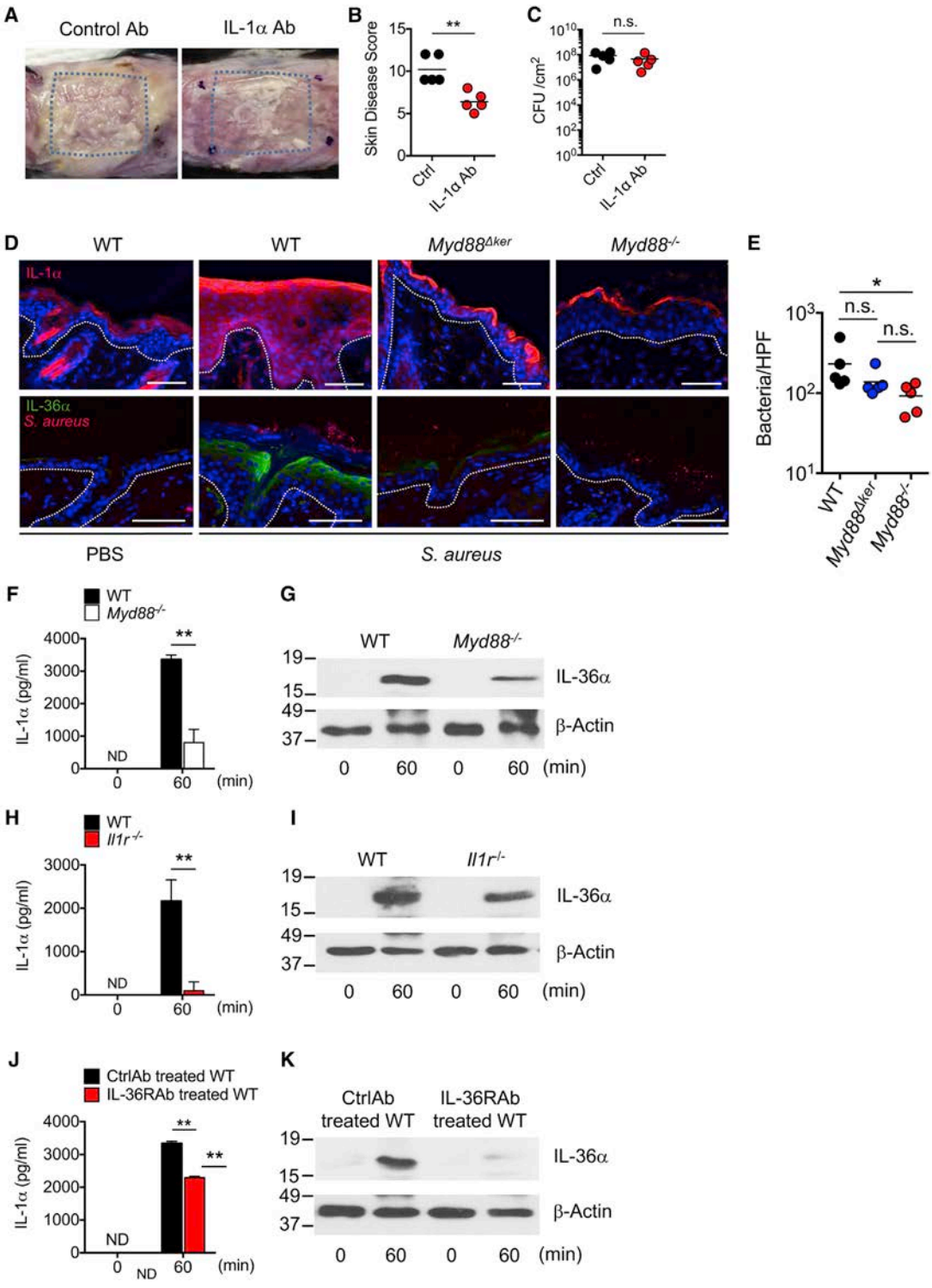

Figure 3. IL-1α and IL-36α Are Induced by *S. aureus* via Myd88 Signaling in Keratinocytes

(A) WT mice treated with IL-1α blocking antibody (IL-1αAb) or isotype-matched control Ab were epicutaneously colonized with *S. aureus*. Representative macroscopic images of mice colonized with *S. aureus* (n = 5 mice per group).

(B and C) Skin disease scores (B) and *S. aureus* CFUs in the skin (C) of WT mice treated with IL-1α Ab or control Ab. Each dot represents a mouse.

(D) Skin tissues of WT and *Myd88*$^{\Delta ker}$ and *Myd88*$^{-/-}$ mice colonized with *S. aureus* or treated with PBS were stained with Hoechst stain (blue) and antibody against IL-1α (red) or Hoechst stain (blue) and antibodies against *S. aureus* (red) and IL-36α (green). Scale bars, 50 μm.

(E) The numbers of *S. aureus* per high power field (HPF) are shown. Each dot represents average results from an individual mouse.

(legend continued on next page)

(Nakamura et al., 2013), we asked whether PSMα is important for eliciting skin inflammation. We first assessed whether PSMα is important for the release of IL-1α and IL-36α by incubating primary mouse KCs with culture supernatants from WT or isogenic *S. aureus* mutant strains deficient in PSMα1−4 (*psmα*) or PSMβ1−2 (*psmβ*). Incubation with the culture supernatant from WT and mutant *psmβ*, but not mutant *psmα*, strain induced cell death and release of IL-1α and IL-36α (Figures 6A–6C, S5A, and S5B). In addition to mouse KCs, human KCs showed a marked release of IL-1α and enhanced cytotoxicity after treatment with supernatants from the WT and mutant *psmβ* strains, but not mutant *psmα* (Figures S6A and S6B). Complementation of mutant *Δpsmα S. aureus* with *psmα* plasmid restored cytotoxicity and cytokine release in both mouse and human KCs (Figures S5F, S5G, S6C, and S6D). In addition, stimulation of human KCs with synthetic PSMα3 peptide induced IL-1α release and cytotoxicity (Figures S6E and S6F). Furthermore, pre-treatment with anakinra, an IL-1R antagonist, reduced IL-1α release induced by PSMα3 peptide without affecting cytotoxicity (Figures S6G and S6H). To determine whether PSMα is important for the induction of skin inflammation, we infected WT mice with WT and mutant *psmα* and *psmβ S. aureus* strains using the epicutaneous model. Histological analysis revealed that the ability of the *psmα* mutant strain to induce skin disease, epidermal thickening, and neutrophil infiltration was impaired compared with the WT and *psmβ* strains (Figures 6D, 6E, 6G, 6H, S5C, and S5D). There were reduced pathogen loads and IL-17 production in the skin of mice infected with the *psmα* mutant strain when compared to the WT bacterium (Figures 6F, 6I, and S5E). These results suggest that PSMα from *S. aureus* is a key virulence factor for triggering *S. aureus*-induced skin inflammation.

DISCUSSION

The mechanism by which *S. aureus* triggers skin inflammation in response to epidermal colonization has remained largely unknown. The paucity of knowledge is largely explained by the lack of a model of epidermal infection that mimics the virulent lifestyle of the pathogen. Using conditions in which *S. aureus* colonizes the epidermis and induces virulence (Nakamura et al., 2013), we found that Agr-regulated PSMα peptides trigger the release of the KC alarmins IL-1α and IL-36α to elicit skin inflammation. These observations indicate that the host senses the pathogen indirectly through the induction of KC damage triggered by cytotoxic virulence factors. Because expression of aggressive, cytolytic PSMs appears to be limited mostly to *S. aureus* in comparison to *S. epidermidis* (Cheung et al., 2010), these observations suggest a model in which the host immune system can discriminate a virulent pathogen from commensals by sensing KC damage. Because PSMα peptides have been shown to induce chemokines from neutrophils (Kretschmer et al., 2010; Wang et al., 2007), it is possible that PSMα also contributes to skin inflammation via KC-independent mechanisms. Previous work showed that epidermal colonization of some commensals can induce IL-17-producing $CD8^+$ cells in the dermis (Naik et al., 2012, 2015). However, colonization by commensals does not trigger overt inflammation and skin pathology, including epidermal thickening and neutrophil recruitment, which are observed after *S. aureus* infection (Naik et al., 2015). This model also implies that the host senses not the bacterium per se, but the virulent state of the pathogen through PSMα-induced release of KC alarmins. *S. aureus* lacking PSMα peptides accumulates at lower numbers than the WT bacterium after epicutaneous colonization. These results suggest that the pathogen benefits from PSMα production, at least in part, by inducing the release of nutrients or other factors from damaged KCs.

These studies have revealed a critical role for Myd88 signaling within KCs in orchestrating cutaneous inflammation induced by epicutaneous *S. aureus* infection. In mice with deletion of Myd88 in KCs, there was reduction of skin inflammation that was associated with impaired production of IL-1α and IL-36α by KCs. Studies *in vivo* and *in vitro* revealed that IL-1α and IL-36α expression is enhanced by *S. aureus* stimulation and that this is impaired in *Myd88*$^{-/-}$ KCs. Furthermore, induction of IL-1α and IL-36α by *S. aureus* was reduced by IL-1R deficiency and IL-36R neutralization, respectively. These results suggest that IL-1α and IL-36α act in a positive feedback loop that regulates their own expression via Myd88 signaling in KCs. These studies do not rule out a role for Myd88 on cells other than KCs to regulate the inflammatory response in the skin. IL-1R and IL-36R are expressed on immune cells and regulate the differentiation of IL-17-producing immune cells, including γδ T cells, Th17 cells, and ILC3s (Klose and Artis, 2016; Tortola et al., 2012; Vigne et al., 2011). Therefore, it is likely that Myd88 also acts critically on immune cells via IL-1R and IL-36R stimulation to regulate the induction of IL-17-producing cells in response to *S. aureus* epidermal infection. The phenotype of *Myd88*$^{-/-}$ mice is in contrast to that observed in the intradermal *S. aureus* inoculation model in which Myd88 deficiency is associated with increased pathogen loads and tissue pathology (Miller et al., 2006). The opposite roles of Myd88 in the two models suggest a different function of the immune system in the presence and absence of pathogen invasion. In the epicutaneous model, the epidermal barrier is not physically breached, and epidermal thickening and the recruitment of neutrophils are induced via Myd88 function to prevent pathogen invasion. In contrast to the role of Myd88 signaling in sensing pathogen on the epidermal surface in the epicutaneous model, in the dermal/subcutaneous model Myd88 signaling appears more critical in promoting killing of *S. aureus* by immune cells within the dermis and subcutaneous tissues (Miller et al., 2006).

(F–K) IL-1α (F, H, and J) and IL-36α (G, I, and K) release of differentiated primary KCs isolated from WT and *Myd88*$^{-/-}$ mice (F and G), WT and *Il1r1*$^{-/-}$ mice (H and I), or WT mice in the presence of anti-IL-36 neutralizing Mab or isotype-matched control Mab (J and K) and stimulated with culture supernatants of *S. aureus* for indicated time. IL-1α and IL-36α were detected by ELISA assay and immunoblotting, respectively. β-actin in whole-cell lysates is shown as loading control. Data are presented as mean ± SD.

Data are representative of at least two independent experiments. ND, not detected; n.s., not significant; *$p < 0.05$, **$p < 0.01$, by unpaired two-tailed Mann-Whitney U test (B, C, F, H, and J) or Kruskal-Wallis test (E).

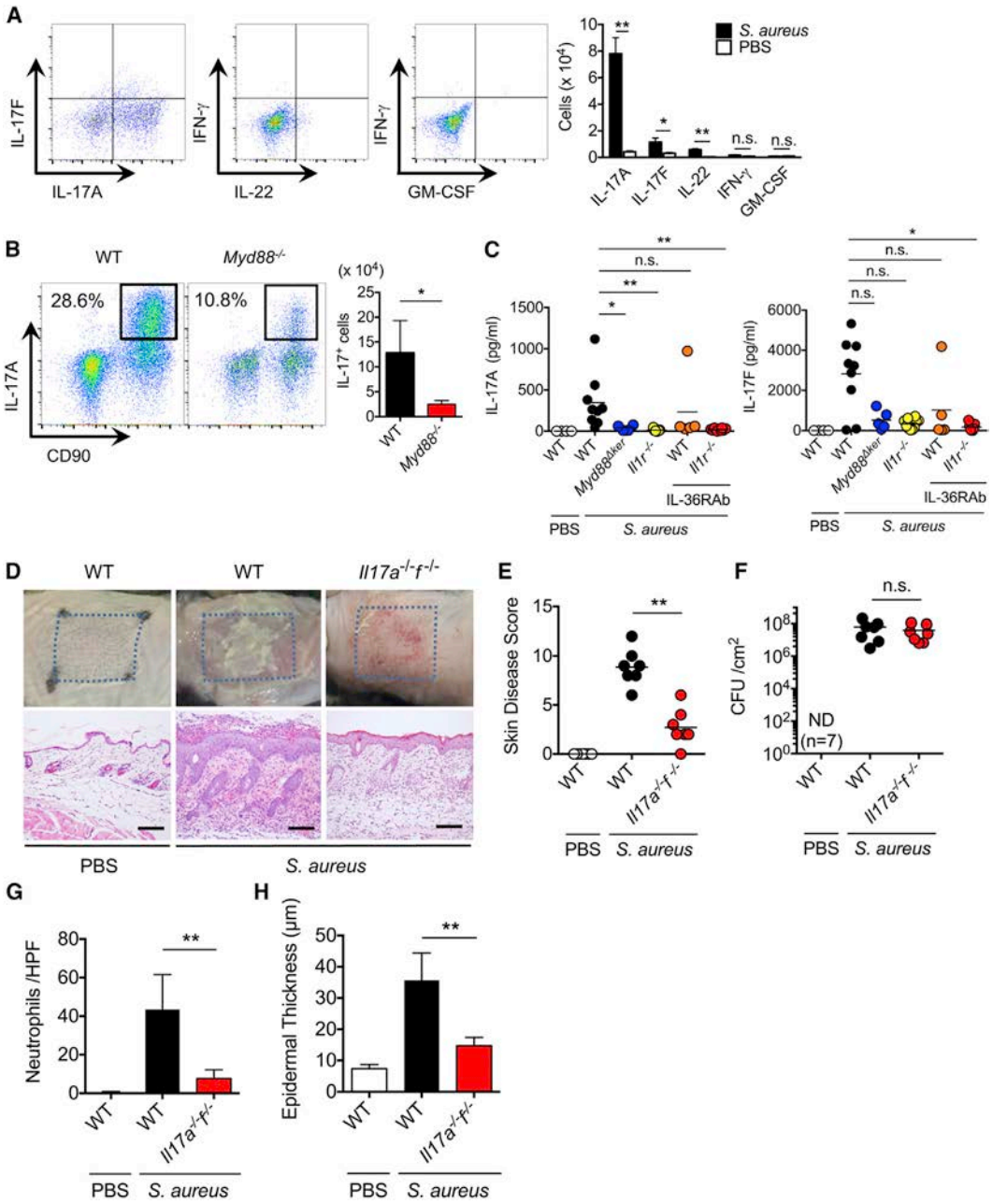

Figure 4. IL-17 Is Critical for Skin Inflammation Induced by Epicutaneous *S. aureus* Colonization

(A) Production of IL-17A, IL-17F, IL-22, IFN-γ, and GM-CSF by skin cells isolated from WT mice colonized with *S. aureus* or treated with PBS. Intracellular cytokine production was assessed in gated CD45$^+$CD90$^+$ cells on day 7 after pathogen colonization by flow cytometry. Representative flow cytometry profiles (left panels) and the number of cytokine-producing cells (right panel). Results in right panel represent mean ± SD of two experiments.

(B) Production of IL-17A by CD45$^+$ cells in the skin of WT and *Myd88*$^{-/-}$ mice 7 days after epicutaneous infection. Representative flow cytometry profiles (left panels) and the number of IL-17A-producing cells (right panel). Results in right panel represent mean ± SD of two experiments.

(C) IL-17A and IL-17F production in skin tissues of WT mice, *Myd88*$^{\Delta ker}$ mice, *Il1r*$^{-/-}$ mice, WT mice treated with IL-36RAb, and *Il1r*$^{-/-}$ mice treated with IL-36RAb 7 days after pathogen colonization. WT mice treated with PBS are shown for comparison. Each dot represents a mouse. Data represent combined data of three independent experiments.

(D) WT and *Il17a*$^{-/-}$*f*$^{-/-}$ mice were epicutaneously colonized with *S. aureus* or treated with PBS. Representative macroscopic images and HE-stained skin sections 7 days after colonization (n = 7 mice per group). Scale bars, 100 μm.

(E–H) Skin disease scores (E), *S. aureus* CFUs in the skin (F), the numbers of neutrophils (G), and epidermal thickness (H) of WT and *Il17a*$^{-/-}$*f*$^{-/-}$ mice colonized with *S. aureus*. WT mice treated with PBS are shown for comparison. Results shown represent combined data of three independent experiments.

Each dot represents a mouse (C, E, and F). Data are presented as mean ± SD (A, B, G, and H). ND, not detected; n.s., not significant; *p < 0.05, **p < 0.01, by unpaired two-tailed Mann-Whitney U test (A, B, E, F, G, and H) or Kruskal-Wallis test (C).

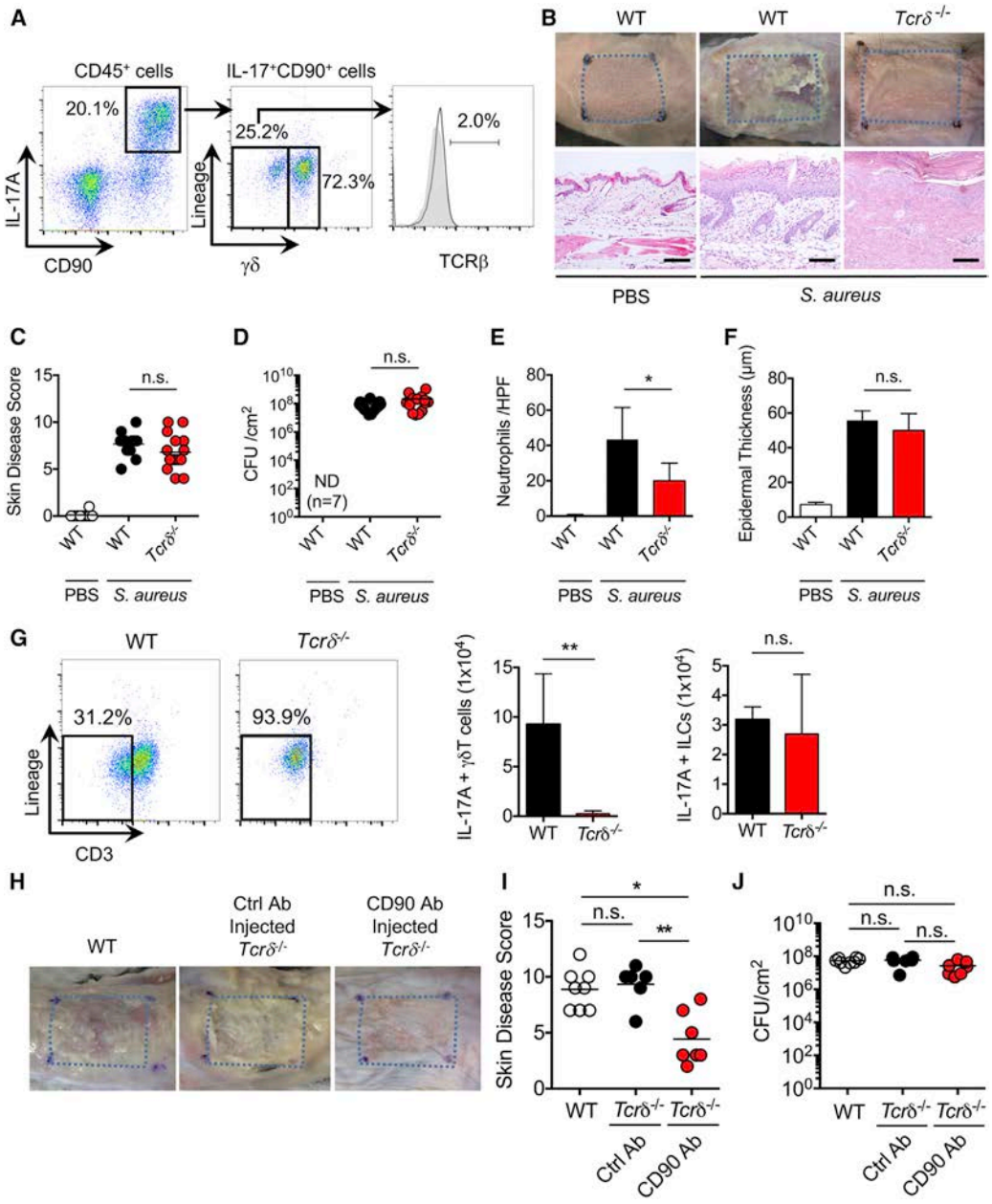

Figure 5. Both γδ T Cells and ILC3s Contribute to Skin Inflammation after *S. aureus* Colonization

(A) IL-17A-producing γδ T cells, ILC3s, and αβ T cells were evaluated by flow cytometric analysis after epicutaneous *S. aureus* colonization. Flow cytometric analysis of lineage (B220, CD11b, CD11c, Gr-1, and NK1.1)-negative cells and γδ T cells was performed on gated $CD45^+CD90^+IL\text{-}17A^+$ skin cells. Data are representative of three independent experiments.

(B) WT and $Tcr\delta^{-/-}$ mice were epicutaneously colonized with *S. aureus*. WT mice treated with PBS are shown for comparison. Representative macroscopic images and HE-stained sections of mouse skin on day 7 after colonization (n = 10–15 mice per group). Scale bars, 100 μm.

(C–F) Day 7 skin disease scores (C), *S. aureus* CFUs in the skin (D), neutrophil numbers in the skin (E), and epidermal thickening (F) of WT and $Tcr\delta^{-/-}$ mice colonized with *S. aureus*. Each dot represents a mouse (C and D). Data are presented as mean ± SD (E and F). Results represent combined data of four independent experiments.

(G) IL-17A-producing γδ T cells and ILC3s were evaluated by flow cytometric analysis in WT and $Tcr\delta^{-/-}$ mice after *S. aureus* colonization. Representative flow cytometric profiles of CD3 and lineage labeling on $CD45^+CD90^+IL\text{-}17A^+$ skin cells (left panels). The number of $IL\text{-}17A^+$ γδ T cells and ILC3s in WT and $Tcr\delta^{-/-}$ mice (right panels). Results in right panels represent mean ± SD of three experiments.

(H) WT mice and $Tcr\delta^{-/-}$ mice treated with control Ab and anti-CD90 Ab were epicutaneously colonized with *S. aureus*. Representative macroscopic images and HE-stained sections of mouse skin on day 7 after colonization (n = 7 mice per group). Scale bars, 100 μm.

(I and J) Skin disease scores (I) and *S. aureus* CFUs in the skin (J) of WT mice and $Tcr\delta^{-/-}$ mice treated with control Ab and anti-CD90 Ab. Each dot represents a mouse. Results represent combined data of two independent experiments.

ND, not detected; n.s., not significant; *$p < 0.05$, **$p < 0.01$, by unpaired two-tailed Mann-Whitney U test (C–G) or by one-way ANOVA test with Bonferroni's correction (I and J).

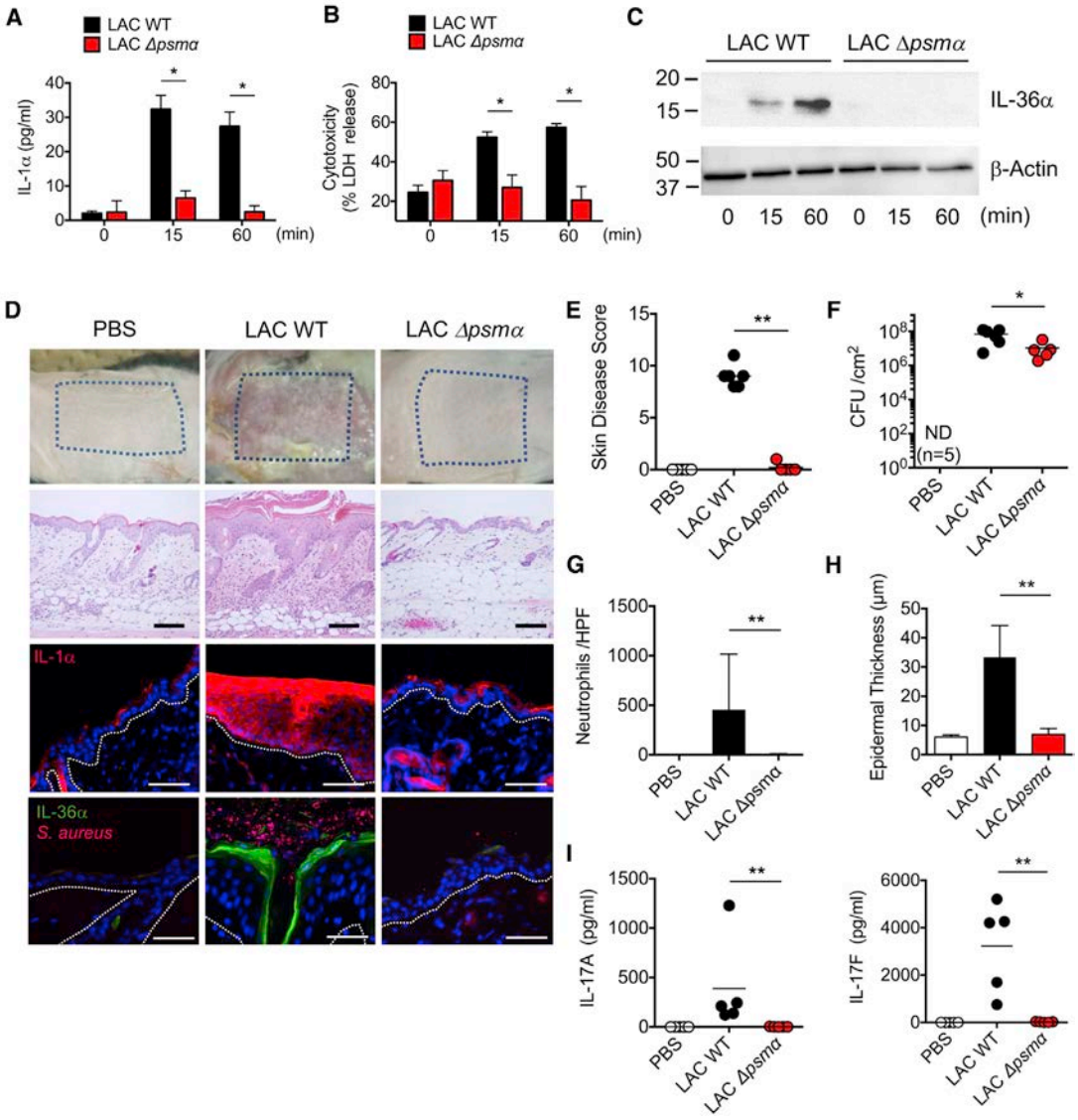

Figure 6. PSMα Peptides Induce the Release of Keratinocyte IL-1α and IL-36α to Mediate Skin Inflammation

(A and B) IL-1α release (A) and cytotoxicity (B) of primary KCs from WT mice stimulated with culture supernatant of WT and *Δpsma S. aureus* (LAC strain) for indicated time. Data are presented as mean ± SD.

(C) IL-36α release from primary KCs stimulated with culture supernatant of WT or *Δpsma S. aureus* for indicated time. IL-36α was detected by immunoblotting. β-actin in whole-cell lysates is shown as loading control.

(D) Representative macroscopic images (top panels) and HE-stained sections (middle upper panels), and sections stained with Hoechst stain (blue) and antibody against IL-1α (red) (middle lower panels) and stained with Hoechst stain (blue) and antibodies against *S. aureus* (red) and IL-36α (green) (bottom panels) of the skin from WT mice colonized with WT and *Δpsma S. aureus* or treated with PBS (n = 5–8 per group) 7 days post-infection. Epidermis/dermis border is marked by dotted white line. Scale bars, 100 (middle upper panels), 50 (middle lower panels), and 25 μm (bottom panels).

(E–H) Day 7 skin disease scores (E), *S. aureus* CFUs in the skin (F), quantification of neutrophils in the lesional skin (G), and epidermal thickening (H) of WT mice colonized with WT and *Δpsma S. aureus* or treated with PBS (n = 5–8 per group). Each dot represents a mouse (E and F). Data are presented as mean ± SD (G and H).

(I) The amounts of IL-17A and IL-17F in the lesional skin of WT mice colonized with WT or *Δpsma S. aureus* or WT mice treated with PBS for 7 days. Each dot represents a mouse.

Data are representative of three independent experiments (A–D). Results represent combined data of three independent experiments (E–I). ND, not detected; n.s., not significant; *p < 0.05, **p < 0.01, by unpaired, two-tailed Mann-Whitney U test.

Our findings may have relevance to the inflammatory response associated with some forms of dermatitis in humans. Over 90% of atopic dermatitis (AD) patients are colonized in the lesional epidermis by *S. aureus*, which is increased during disease flares (Kong et al., 2012; Rudikoff and Lebwohl, 1998). In adult AD, lesional skin has been associated with Th2, Th22, and Th17 immune polarization, but strong Th17 polarization is characteristic of new-onset pediatric AD (Esaki et al., 2016). Previous work

revealed the expression of *S. aureus* Agr virulence factors that produce PSMs in the lesional skin in AD (Nakamura et al., 2013). Furthermore, δ-toxin promoted allergic skin disease through the activation of mast cells that induce Th2 type inflammation in the epicutaneous *S. aureus* model (Nakamura et al., 2013). Together, these results indicate that different PSMs contribute to *S. aureus*-induced skin IL-17-driven inflammation via different mechanisms. However, a role for PSMα-induced IL-1α and IL-36 production in the pathogenesis of AD remains to be investigated. Although there is no direct evidence of *S. aureus* colonization in psoriasis, both psoriasis vulgaris and generalized pustular psoriasis are associated with increased production of IL-1 and IL-36 cytokines (D'Erme et al., 2015; Johnston et al., 2017). The importance of IL-36 in generalized pustular psoriasis is highlighted by the observation that missense loss-of-function mutations of the IL-36R antagonist lead to unrestrained IL-36 activity, which is associated with the disease (Marrakchi et al., 2011). Furthermore, neutralization of IL-17 is highly effective in the treatment of psoriasis (McInnes et al., 2015; Papp et al., 2012). Thus, IL-17 appears to promote protective immunity against pathogens, but if its production is excessive can also contribute to inflammatory disease. Our observations suggest that identifying pathogenic stimuli that trigger KC damage would help unravel the mechanisms underlying the induction of pathogenic IL-17 responses often associated with psoriasis.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.chom.2017.10.008.

AUTHOR CONTRIBUTIONS

S.N., M.M., G.N., and Y.N. designed experiments; S.N., M.M., Y.K., R.O., S.W., T.N., S.S., and Y.N. performed experiments; M.O. provided critical reagents and scientific insight; S.N., M.M., N.I., H.M., G.N., and Y.N. analyzed data; and S.N., M.M., G.N., and Y.N. wrote the paper.

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REFERENCES

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity *9*, 143–150.

Balasubramanian, D., Harper, L., Shopsin, B., and Torres, V.J. (2017). Staphylococcus aureus pathogenesis in diverse host environments. Pathog. Dis. *75*, https://doi.org/10.1093/femspd/ftx005.

Bikle, D.D., Xie, Z., and Tu, C.L. (2012). Calcium regulation of keratinocyte differentiation. Expert Rev. Endocrinol. Metab. *7*, 461–472.

Cheung, G.Y., Rigby, K., Wang, R., Queck, S.Y., Braughton, K.R., Whitney, A.R., Teintze, M., DeLeo, F.R., and Otto, M. (2010). Staphylococcus epidermidis strategies to avoid killing by human neutrophils. PLoS Pathog. *6*, e1001133.

Cheung, G.Y., Joo, H.S., Chatterjee, S.S., and Otto, M. (2014). Phenol-soluble modulins–critical determinants of staphylococcal virulence. FEMS Microbiol. Rev. *38*, 698–719.

D'Erme, A.M., Wilsmann-Theis, D., Wagenpfeil, J., Hölzel, M., Ferring-Schmitt, S., Sternberg, S., Wittmann, M., Peters, B., Bosio, A., Bieber, T., and Wenzel, J. (2015). IL-36γ (IL-1F9) is a biomarker for psoriasis skin lesions. J. Invest. Dermatol. *135*, 1025–1032.

Esaki, H., Brunner, P.M., Renert-Yuval, Y., Czarnowicki, T., Huynh, T., Tran, G., Lyon, S., Rodriguez, G., Immaneni, S., Johnson, D.B., et al. (2016). Early-onset pediatric atopic dermatitis is TH2 but also TH17 polarized in skin. J. Allergy Clin. Immunol. *138*, 1639–1651.

Franchi, L., Kamada, N., Nakamura, Y., Burberry, A., Kuffa, P., Suzuki, S., Shaw, M.H., Kim, Y.G., and Núñez, G. (2012). NLRC4-driven production of IL-1β discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. Nat. Immunol. *13*, 449–456.

Gresnigt, M.S., and van de Veerdonk, F.L. (2013). Biology of IL-36 cytokines and their role in disease. Semin. Immunol. *25*, 458–465.

Horai, R., Asano, M., Sudo, K., Kanuka, H., Suzuki, M., Nishihara, M., Takahashi, M., and Iwakura, Y. (1998). Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J. Exp. Med. *187*, 1463–1475.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. *162*, 3749–3752.

Hou, B., Reizis, B., and DeFranco, A.L. (2008). Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity *29*, 272–282.