



Staphylococcus aureus proteases trigger eosinophil-mediated skin inflammation

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Staphylococcus aureus skin colonization and eosinophil infiltration are associated with many inflammatory skin disorders, including atopic dermatitis, bullous pemphigoid, Netherton's syndrome, and prurigo nodularis. However, whether there is a relationship between *S. aureus* and eosinophils and how this interaction influences skin inflammation is largely undefined. We show in a preclinical mouse model that *S. aureus* epicutaneous exposure induced eosinophil-recruiting chemokines and eosinophil infiltration into the skin. Remarkably, we found that eosinophils had a comparable contribution to the skin inflammation as T cells, in a manner dependent on eosinophil-derived IL-17A and IL-17F production. Importantly, IL-36R signaling induced CCL7-mediated eosinophil recruitment to the inflamed skin. Last, *S. aureus* proteases induced IL-36 α expression in keratinocytes, which promoted infiltration of IL-17-producing eosinophils. Collectively, we uncovered a mechanism for *S. aureus* proteases to trigger eosinophil-mediated skin inflammation, which has implications in the pathogenesis of inflammatory skin diseases.

eosinophils | inflammatory skin diseases | *Staphylococcus aureus* | interleukin-17 | interleukin-36

Staphylococcus aureus is the leading cause of skin and soft tissue infections in the United States (1), which is exacerbated by the emergence of antibiotic-resistant clinical isolates such as community-acquired methicillin-resistant *S. aureus* (MRSA) (2). Furthermore, *S. aureus* skin colonization is associated with various inflammatory skin diseases. For instance, the abundance of *S. aureus* on the skin of atopic dermatitis (AD) patients correlates with disease severity (3), with highly toxicogenic *S. aureus* strains dominating on the skin (4). Importantly, *S. aureus*-derived virulence factors are associated with AD skin inflammation, including phenol soluble modulin- α , proteases, superantigens, and delta toxin (5–8). A recent study found that *S. aureus* was predominant in the microbiome of prurigo nodularis (PN) patients, which was highly similar to AD patients (9). In bullous pemphigoid (BP), *S. aureus* colonization was observed in 85% of patients and antibiotic treatment resulted in clinical improvement (10, 11). Similarly, Williams et al. found that Netherton's syndrome (NS) patients have a microbiome dominated by staphylococci, including *S. aureus*, which contributed to skin inflammation (7). However, a better understanding of the complex interactions between *S. aureus* and host responses in inflammatory skin disease pathogenesis is needed for the development of novel therapeutics.

Eosinophils are myeloid-derived granulocytes that are involved in various biological functions, including wound healing, host defense against parasitic infection, and allergic inflammation (12–15). Patients with AD have significantly increased numbers of eosinophils in both the blood and the skin that correlates with disease severity (13, 16–19). Moreover, the presence of *S. aureus* on the lesional skin of AD patients correlates with significantly greater eosinophil numbers in circulation (19). Similarly, BP patients have an increase in activated eosinophils in the blood and skin (20), including a significant correlation between dermal eosinophilia and disease severity (20, 21). Moreover, NS is associated with eosinophilia and eosinophilic esophagitis (22, 23), whereas PN is found to have increased eosinophil granule deposition in lesional skin (20–25).

Despite the well-characterized presence of *S. aureus* and eosinophils in various inflammatory skin conditions, how their interaction influences skin inflammation is unclear. Moreover, the mechanisms by which *S. aureus* promotes eosinophil infiltration into the skin are not entirely known. In this study, we used a mouse model of *S. aureus* epicutaneous (e.c.) exposure to determine the contribution of eosinophils to skin inflammation using wild-type (WT) and eosinophil-deficient (*Eos*^{−/−}) mice (5, 6, 26). Furthermore, we elucidated the host and bacterial-derived mechanisms that regulate eosinophil-mediated skin inflammation, which may provide additional therapeutic targets to treat inflammatory skin disorders.

Significance

Staphylococcus aureus is the leading cause of skin and soft tissue infections in the United States, which is exacerbated by the emergence of antibiotic-resistant clinical isolates. *S. aureus* skin colonization and eosinophil infiltration are associated with many inflammatory skin disorders, including atopic dermatitis, bullous pemphigoid, Netherton's syndrome, and prurigo nodularis. However, whether there is a relationship between *S. aureus* and eosinophils and how this interaction influences skin inflammation is largely undefined. The significance of this study is that it describes a mechanism by which eosinophils contribute to skin inflammation in response to *S. aureus* skin exposure. These findings may provide potential therapeutic targets for the treatment of inflammatory skin disorders and potentially other diseases.

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Results

Eosinophils Contribute to Skin Inflammation. To investigate the interactive role of *S. aureus* and eosinophils in skin inflammation, a mouse model of *S. aureus* e.c. exposure was used by securing a *S. aureus*-soaked gauze pad (1×10^8 CFU/100 μ L, MRSA strain LAC4303) to the depilated dorsal skin of mice with adhesive bandages for 7 d (5, 6, 26). We focused on day 7 as it had the most marked increase in disease scoring, epidermal thickening, and immune cell infiltration (e.g., eosinophils) for the time points tested (SI Appendix, Fig. S1). Since *S. aureus* skin colonization and eosinophil infiltration are observed in various inflammatory skin disorders (7, 19, 20, 22, 24), we first set out to determine whether *S. aureus* skin exposure induced eosinophil infiltration

into the skin. We observed a significant increase in eosinophils in *S. aureus*-infected skin (day 7) compared to naive skin (day 0) (Fig. 1A). Moreover, there was markedly increased expression of the eosinophil-attracting chemokines *Ccl7* (MCP-3) and *Ccl8* (MCP-2) in the skin, whereas expression of *Ccl11* (Eotaxin-1) and *Ccl24* (Eotaxin-2) were significantly decreased or similar between the groups as for *Il5* (Fig. 1B).

To determine whether eosinophils contributed to skin inflammation, we performed our *S. aureus* e.c. exposure model on BALB/c WT and eosinophil-deficient C.129S1(B6)-*Gata1*^{tm6Sbs} (*Eos*^{-/-}) or naive WT mice. As expected, naive WT mice had no skin inflammation (e.g., erythema, scaling, swelling), which was significantly induced upon *S. aureus* exposure (Fig. 1C and D). We found that *Eos*^{-/-} mice had significantly reduced skin inflammation (e.g.,

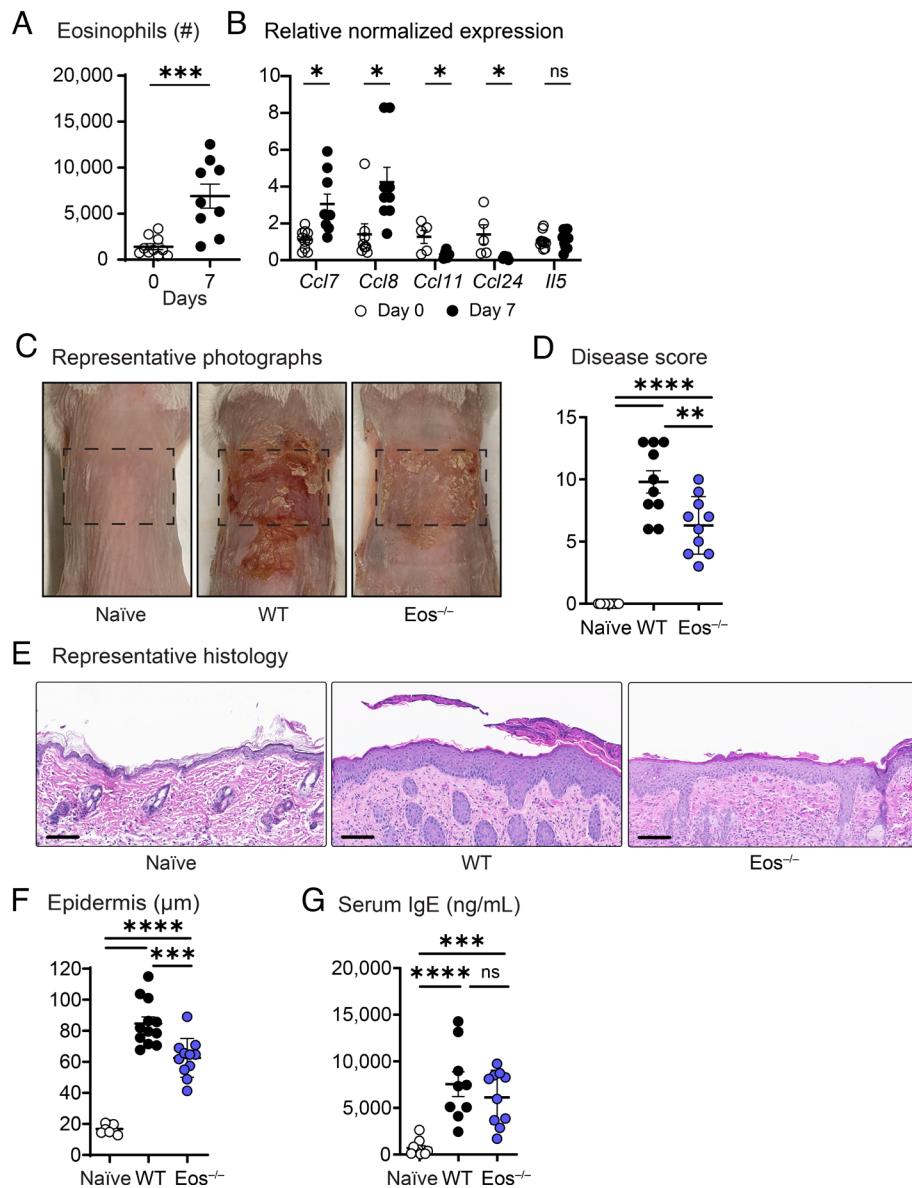


Fig. 1. *S. aureus* induces eosinophil-mediated skin inflammation. (A and B) WT female mouse skin was naive (day 0) or e.c. exposed to *S. aureus* (1×10^8 CFU) for 7 d (day 7), and a 10-mm skin biopsy was harvested. (A) Mean numbers of eosinophils (\pm SEM) in the skin as measured by FACS ($n \geq 7$ per group). (B) Relative normalized gene expression (\pm SEM) in the skin, normalized to β -actin in naive skin ($n \geq 6$ per group). (C–F) WT and *Eos*^{-/-} female mice were e.c. exposed to *S. aureus* (1×10^8 CFU) on the dorsal skin for 7 d, and a 10-mm skin biopsy was harvested ($n \geq 9$ per group). (C) Representative skin photographs. (D) Mean disease score, scored based on the sum of three individual grades for erythema, edema, and scaling/erosion for a total range of 0 to 13 (\pm SEM). (E) Representative histology (H&E stain, 200 \times magnification). (F) Mean epidermal thickness (\pm SEM). (G) Mean serum IgE levels (ng/mL) (\pm SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ as calculated by two-tailed Student's *t* test (A and B) or one-way ANOVA multiple comparisons test with Tukey correction (D, F, and G). Scale bars = 100 μ m. Data are combined from at least two independent experiments.

erythema, scaling, swelling) compared to WT mice, as measured by the disease score (Fig. 1 C and D). Skin inflammation was further assessed by H&E (hematoxylin and eosin)-stained skin sections, which revealed a marked decrease in epidermal thickening in Eos^{-/-} mice compared to WT mice (Fig. 1 E and F), yet significantly greater than naive skin. However, although IgE levels were induced compared to naive mice, there were no significant differences in serum IgE levels between WT and Eos^{-/-} mice (Fig. 1 G). To confirm eosinophil deficiency and that there were no defects in other myeloid cells in the Eos^{-/-} mice, as previously described (27, 28), we performed flow cytometric analysis of the blood and skin of WT and Eos^{-/-} mice. We found that Eos^{-/-} mice had a specific deficiency in eosinophils, but not other myeloid or lymphocytic cells (*SI Appendix*, Fig. S2). We further confirmed the eosinophil phenotype by depletion of eosinophils in WT mice using an anti-CCR3 mAb or isotype control. We found a modest, yet statistically significant decrease in disease scores and eosinophils in the anti-CCR3-treated mice compared to isotype controls (*SI Appendix*, Fig. S3 A–C). We also performed an adoptive transfer experiment, whereby Eos^{-/-} mice were injected 1 h prior to e.c. exposure with 2×10^6 eosinophils or PBS as a control. We observed a trend toward increased disease scores in Eos^{-/-} mice i.v. injected with eosinophils compared to PBS controls (*SI Appendix*, Fig. S3 D and E). Taken together, these results indicated that eosinophils contributed to the skin inflammation upon *S. aureus* exposure.

Eosinophils Promote the IL-17 Pathway during Skin Inflammation.

To further define the role of eosinophils in skin inflammation, we performed RNA-seq analysis on day 7 skin from WT and Eos^{-/-} mice. Consistent with the differences in skin inflammation, principal component analysis (PCA) showed distinct population clustering between WT and Eos^{-/-} mice (Fig. 2A). We performed xCell enrichment analyses to infer differences in the cellular composition in the skin, which revealed an overall marked decrease in ImmuneScore with concomitant decreases in signatures for mesenchymal stem cells (MSCs), basophils, and neutrophils in Eos^{-/-} mice compared to WT mice (Fig. 2 B and C). In contrast, there were notable increases in the signatures for keratinocytes, preadipocytes, and hematopoietic stem cells (HSCs) in Eos^{-/-} mice compared to WT mice.

Gene set enrichment analysis (GSEA) of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways revealed that the IL-17, chemokine, and Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathways were significantly down-regulated in the skin of Eos^{-/-} mice compared to WT mice, which are immune pathways associated with inflammatory skin conditions (e.g., AD, BP, PN, and NS) (7, 19, 20, 22, 24, 29–32) (Fig. 2D). Since we and others have previously identified a role for IL-17 in the skin inflammation in this model (5, 6), we further examined the down-regulated genes in the IL-17 pathway, which included *Mmp3*, *Tnfa*, *Il1b*, *Il17a*, *Il13*, *Il6*, and *Ccl7* (Fig. 2E). Expression of differentially expressed genes (DEGs) was confirmed by qPCR for *Mmp3*, *Tnfa*, *Il1b*, and *Il6* (Fig. 2F). Taken together, our RNA-seq analysis revealed an important role for eosinophils in regulating expression of immune pathways during skin inflammation.

Eosinophils Are a Predominant Source of IL-17A and IL-17F during Skin Inflammation. We previously reported that T cell-derived IL-17A and IL-17F promoted skin inflammation upon *S. aureus* e.c. exposure (5). Given that our RNA-seq analysis indicated that eosinophils were associated with the IL-17 pathway, we set out to determine whether eosinophils directly contributed to IL-17A and IL-17F production in the inflamed skin. To this end, we used *in vivo* fluorescent imaging with the IL-17A-tdTomato/IL-17F-GFP

dual-color reporter mouse to quantify IL-17A and IL-17F levels in the skin, which we have previously shown to correlate with IL-17A and IL-17F protein production (33). We found that *S. aureus* e.c. exposure induced significant IL-17A and IL-17F expression in the skin compared to naive mice (Fig. 3 A and B). Next, we performed flow cytometric analysis of cells from the inflamed skin to identify the IL-17A (tdTomato+) and IL-17F (GFP+) producing cells (*SI Appendix*, Fig. S4A) and the frequency of IL-17A (tdTomato+) and IL-17F (GFP+) expression among immune cells (*SI Appendix*, Fig. S4B). Remarkably, eosinophils were a predominant source of IL-17A and IL-17F in the inflamed skin, with levels exceeding or comparable to the IL-17-producing T cell population (Fig. 3C and *SI Appendix*, Fig. S5A). We confirmed the eosinophil-derived IL-17A cytokine production using immunofluorescent histology on day 7 inflamed skin in WT mice (Fig. 3D). Furthermore, we found similar ratios of tdTomato+ or IL-17A+ eosinophils per total eosinophils in the day 7 inflamed skin by flow cytometry in IL-17A-tdTomato/IL-17F-GFP dual reporter mice (~20%) and by immunofluorescent histology in WT mice (~30%) (*SI Appendix*, Fig. S5 B and C).

Since eosinophils and T cells were the main IL-17A- and IL-17F-producing cells in the inflamed skin, we next determined their differential contributions to skin inflammation. To this end, we performed our *S. aureus* e.c. exposure model on BALB/c WT, Eos^{-/-}, Rag1^{-/-} (lacking mature B and T cells), and Eos×Rag1^{-/-} (lacking mature B and T cells, and eosinophils) mice. We found that skin inflammation as measured by the disease score was markedly reduced in Eos^{-/-}, Rag1^{-/-}, and Eos×Rag1^{-/-} mice as compared to WT mice, with Eos×Rag1^{-/-} mice having significantly decreased disease scores compared to Eos^{-/-} and Rag1^{-/-} mice (*SI Appendix*, Fig. S6 A and B). Similarly, epidermal thickness was significantly lower in the Eos^{-/-}, Rag1^{-/-} and Eos×Rag1^{-/-} mice compared to WT mice (*SI Appendix*, Fig. S6 C and D). In contrast to the disease score, the Eos^{-/-}, Rag1^{-/-}, and Eos×Rag1^{-/-} mice had similar epidermal thickness measurements. Interestingly, we found that eosinophil trafficking to the inflamed skin was T cell independent, as there was not a decrease, but rather a significant increase in the eosinophil counts of *S. aureus* e.c. exposed Rag1^{-/-} skin compared to WT skin (*SI Appendix*, Fig. S6E). Taken together, we found that eosinophils are a major IL-17A- and IL-17F-producing cell in the *S. aureus* exposed skin and have a similar contribution to the skin inflammation as T cells.

Eosinophil-Derived IL-17A and IL-17F Promote Skin Inflammation.

We next investigated the role of eosinophil-derived IL-17A and IL-17F in the skin inflammation by performing an adoptive transfer experiment, whereby purified eosinophils from IL-5tg mice were injected intravenously into IL-17A/F^{-/-} mice in the presence of anti-IL-17A/F neutralizing mAbs or isotype control mAbs (Fig. 3E). Thus, eosinophils were the only IL-17A- and IL-17F-producing cells in the IL-17A/F^{-/-} mice. Notably, after the eosinophil adoptive transfer, the disease score and epidermal thickness were significantly reduced in the IL-17A/F^{-/-} mice treated with anti-IL-17A/F neutralizing mAbs compared to IL-17A/F^{-/-} mice treated with isotype control mAbs (Fig. 3 F–I). Collectively, our results indicated that eosinophil-derived IL-17A and IL-17F promoted skin inflammation.

IL-36R Signaling Drives Eosinophil Trafficking to the Skin upon *S. aureus* e.c. Exposure. We next wanted to identify which host-derived signals induced eosinophil trafficking to the inflamed skin. Previously, we and others have reported the role of IL-36R signaling in triggering skin inflammation upon *S. aureus* e.c. exposure (5, 6, 26). Thus, we hypothesized that IL-36R signaling was involved

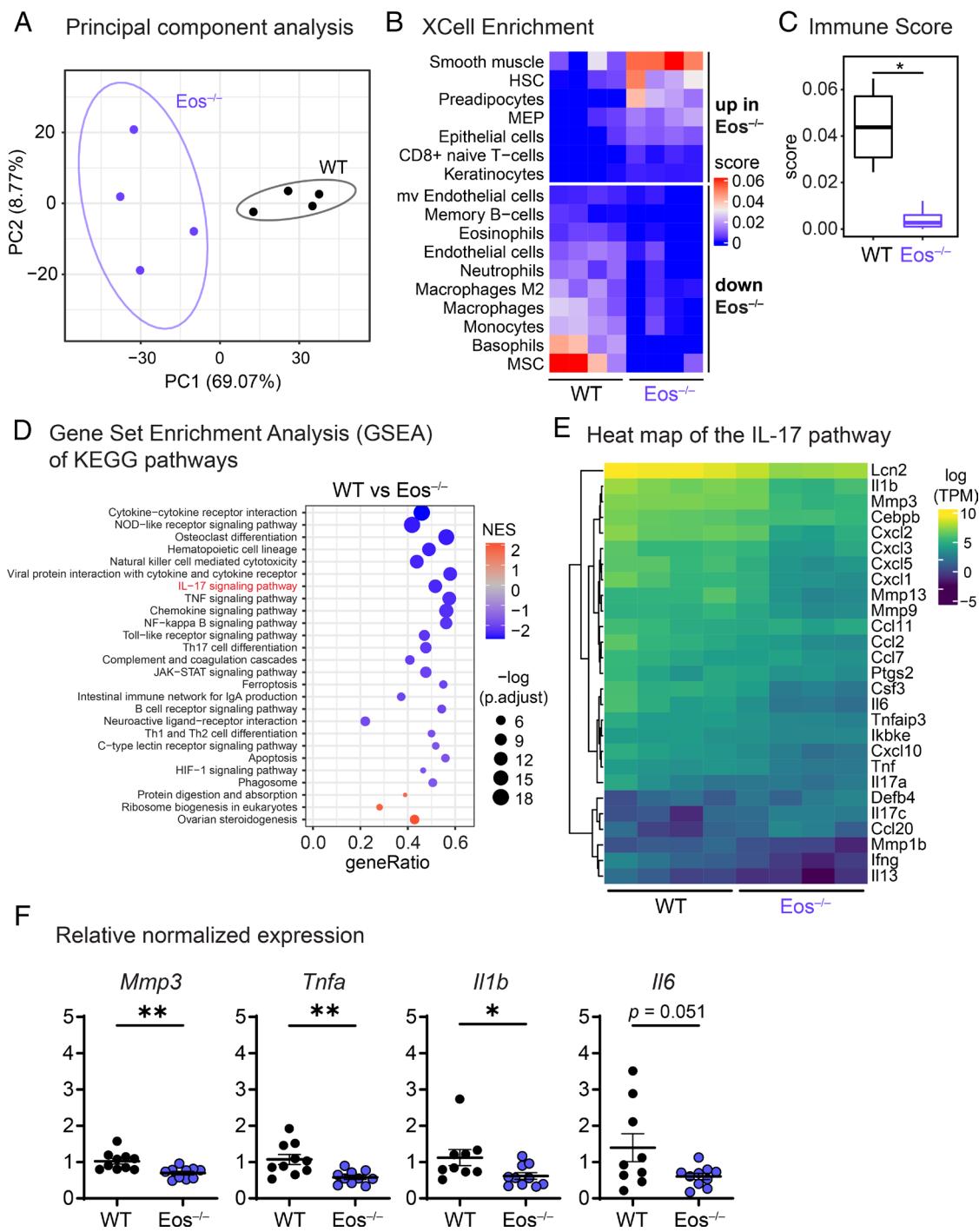


Fig. 2. Eosinophils promote the IL-17 pathway during skin inflammation. WT and $Eos^{-/-}$ female mice were e.c. exposed to *S. aureus* (1×10^8 CFU) on the dorsal skin for 7 d, and a 10-mm skin biopsy was harvested ($n = 4$ per group). (A) PCA of transcripts per million (TPM) values for skin of WT and $Eos^{-/-}$ mice. (B) Cell type enrichment analysis with the xCell algorithm. Displayed are the cell types with significant differences between $Eos^{-/-}$ and WT mice determined by Wilcoxon rank-sum P -value < 0.05 . The Immune Score (C) is the sum of all scores for all immune cell type scores for a given sample. HSC = hematopoietic stem cells, MEP = megakaryocyte-erythroid progenitors, mv = microvascular, MSC = mesenchymal stem cells. (D) GSEA of significant DEGs with gene sets (adjusted P -value < 0.05) from the KEGG. NES = Normalized Enrichment Score (E) Corresponding heat map of the KEGG IL-17 signaling pathway [adjusted P -value < 0.05 and $\text{abs}(\log 2\text{FC}) > 1$]. (F) Relative normalized gene expression ($\pm \text{SEM}$) in the skin, normalized to β -actin in WT skin. * $P < 0.05$ and ** $P < 0.01$ as calculated by two-tailed Student's t test. qPCR data are combined from at least two independent experiments ($n \geq 9$ per group).

in the increased presence of eosinophils in the inflamed skin. As expected, we found that $IL-36R^{-/-}$ mice had markedly reduced disease scores and epidermal thickness compared to WT mice upon *S. aureus* e.c. exposure (Fig. 4 A–D). Notably, $IL-36R^{-/-}$ mice had significantly reduced eosinophils in the inflamed skin compared to WT mice (Fig. 4E), which corresponded to decreased expression of *Ccl7*, *Il17a*, and *Il17f* in the skin (Fig. 4 F and G).

In contrast, expression of the eosinophil-attracting chemokines *Ccl8*, *Ccl11*, and *Ccl24* were either increased or similar between $IL-36R^{-/-}$ and WT mice. Since *Ccl7* transcript correlated with inflammation, we next asked whether CCL7 protein production was associated with the inflamed skin. To this end, we performed immunofluorescent histology on naive and WT inflamed skin and found that *S. aureus* e.c. exposure induced significant

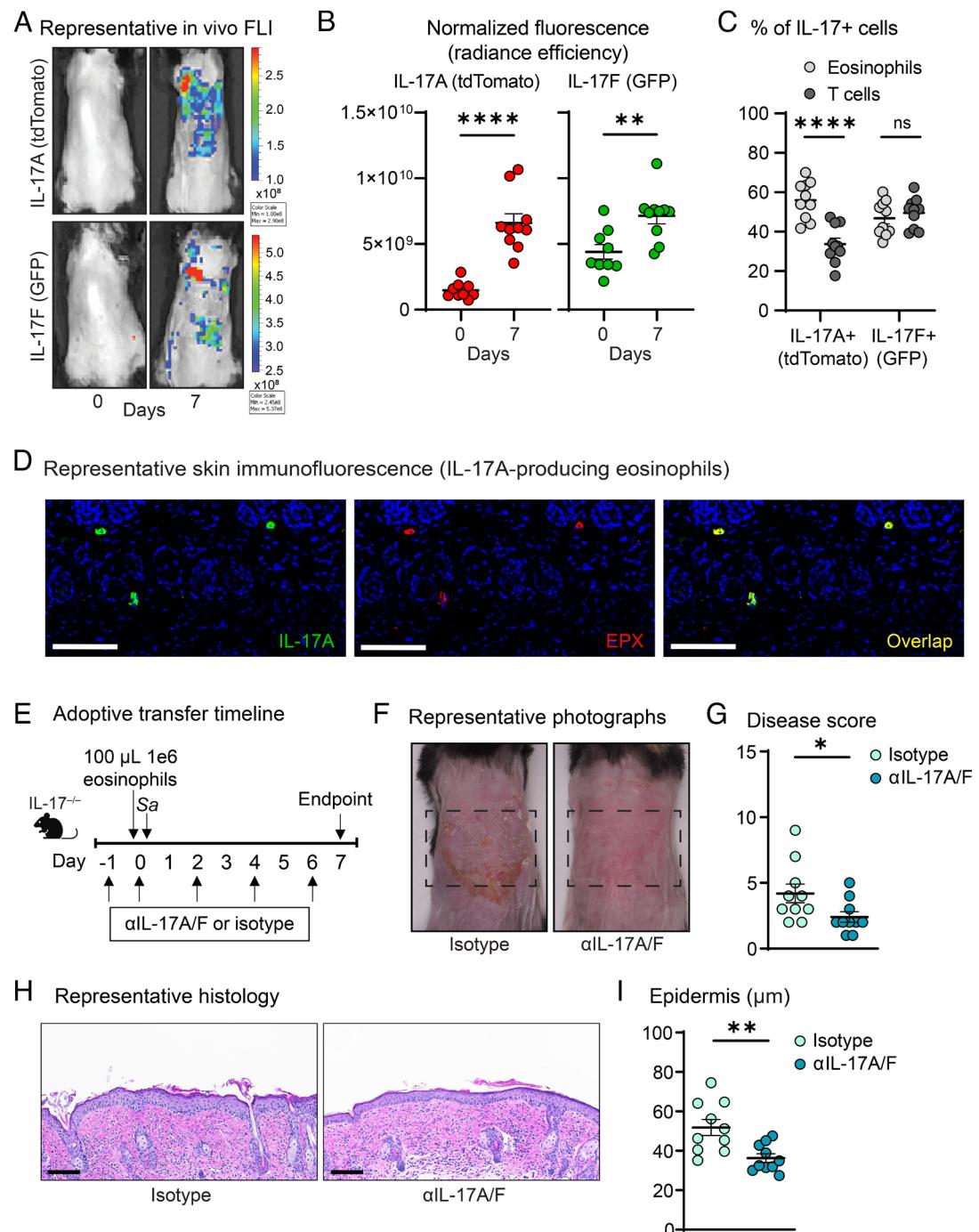


Fig. 3. Eosinophil-derived IL-17A and IL-17F drive skin inflammation. (A–C) IL-17A-tdTomato/IL-17F-GFP dual reporter female mice ($n \geq 9$ per group) were e.c. exposed to *S. aureus* (1×10^8 CFU) on the dorsal skin for 7 d, and a 10-mm skin biopsy was harvested. (A) Representative in vivo fluorescent images (FLI). (B) Mean total flux (photons/s) of IL-17A (tdTomato) and IL-17F (GFP) (\pm SEM). (C) Mean eosinophil and T cell populations as a % of CD45+IL-17+ cells in the skin (\pm SEM). (D) Representative immunofluorescence for IL-17A (green), EPX (eosinophils, red), and DAPI (blue) in day 7 WT *S. aureus*-e.c. infected skin (400 \times magnification) ($n = 8$ images). (E–I) Eosinophils (1×10^6) from female IL-5tg mice were adoptively transferred into female IL-17A/F^{−/−} mice, which were e.c. exposed with *S. aureus* (1×10^8 CFU) for 7 d in the presence of IL-17A/F neutralizing or isotype control mAbs ($n = 10$ per group). (E) Representative photographs. (F) Mean disease score (\pm SEM). (G) Mean epidermal thickness (\pm SEM). (H) Representative histology (H&E stain, 200 \times magnification) and (I) mean epidermal thickness (\pm SEM). ns; not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ as calculated by two-tailed Student's *t* test (B, C, F, and H). Scale bars are 100 μm in (G). Data are combined from at least two independent experiments. (Scale bars are 100 μm .)

CCL7 protein expression in keratinocytes (Fig. 4*H*). Since IL-36R was critical for *Ccl7* transcript expression and IL-36R on keratinocytes is involved in skin inflammation (6, 34), we set out to determine whether IL-36R on keratinocytes was important for the induction of CCL7 protein in the skin. Using IL-36R^{−/−} and keratinocyte-specific IL-36R deficient (K14-IL-36R^{−/−}) mice, we revealed that the induction of CCL7 protein upon *S. aureus* e.c. exposure required the expression of IL-36R on keratinocytes

(Fig. 4*H*). Thus, IL-36R signaling induced eosinophil recruitment to the inflamed skin upon *S. aureus* e.c. exposure, which involved increased CCL7 expression.

CCL7 Contributes to Skin Inflammation and Eosinophil Recruitment Following *S. aureus* e.c. Exposure. Since CCL7 production was associated with skin inflammation in our model and in human AD patients (35, 36), we hypothesized that CCL7

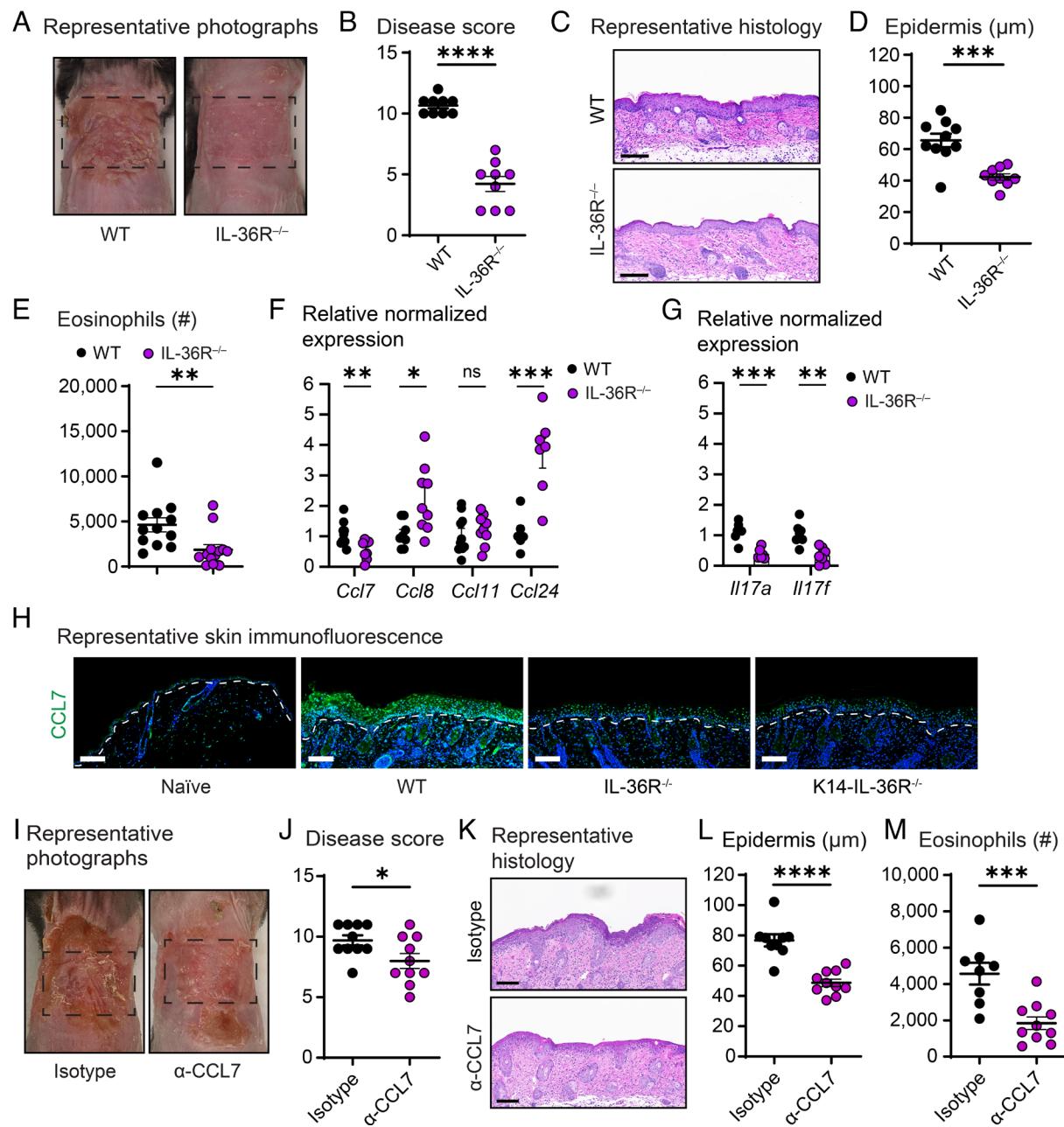


Fig. 4. IL-36R promotes CCL7-mediated eosinophil trafficking to the skin and skin inflammation. (A–G) WT and IL-36R^{-/-} female mice were e.c. exposed to *S. aureus* (1×10^8 CFU) on the dorsal skin for 7 d, and 10-mm skin biopsies were harvested ($n \geq 9$ per group). (A) Representative skin photographs. (B) Mean disease score (\pm SEM). (C) Representative histology (H&E stain, 200 \times magnification). (D) Mean epidermal thickness (\pm SEM). (E) Mean numbers of eosinophils (\pm SEM) in the skin as measured by FACS. Relative normalized expression of (F) eosinophil-related and (G) IL-17A/F genes in the skin (\pm SEM), normalized to β -actin in WT skin. (H) Representative skin immunofluorescence of naïve, WT, IL-36R^{-/-}, and K14-IL-36R^{-/-} skin for CCL7 (green) and DAPI (blue) at 400 \times magnification. The dotted lines represent the epidermal-dermal junction ($n = 4$ stained sections per group). (I–M) WT female mice were e.c. exposed to *S. aureus* (1×10^8 CFU) on the dorsal skin for 7 d and were treated with anti-CCL7 neutralizing antibody (20 μ g) or isotype control mAbs on days 1, 2, and 5 ($n \geq 8$ per group). (I) Representative skin photographs. (J) Mean disease score (\pm SEM). (K) Representative histology (H&E stain, 200 \times magnification). (L) Mean epidermal thickness (\pm SEM). (M) Mean numbers of eosinophils (\pm SEM) in the skin as measured by FACS. n.s.; not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ as calculated by two-tailed Student's *t* test (B, D–G, J, L, and M). Scale bars are 100 μ m in (C, H, and K). Data are combined from at least two independent experiments.

promoted skin inflammation via the recruitment of eosinophils into the skin. To assess this, we treated WT mice e.c. exposed to *S. aureus* with either a neutralizing anti-CCL7 mAb or isotype control mAb. We found that WT mice treated with the anti-CCL7 mAb had a statistically significant reduction in the disease score and epidermal thickness compared to isotype control mAb-treated mice (Fig. 4 I–L), which was associated with markedly reduced eosinophils in the skin (Fig. 4 M). Collectively, these results indicated that CCL7 contributed to the skin inflammation and eosinophil influx into the inflamed skin.

S. aureus Proteases Trigger IL-36 α Production in the Skin. Next, we wanted to unravel how *S. aureus* induced eosinophil responses during skin inflammation. Previous studies have shown that staphylococci-derived proteases are critical for initiating inflammation in mouse and human skin (7, 8). However, the immune mechanisms by which *S. aureus* proteases trigger skin inflammation are largely unknown. To determine how *S. aureus* proteases mediated the skin inflammation, we e.c. challenged WT mice with a *S. aureus* proteases-deficient mutant (Δ proteases), which lacks 10 extracellular proteases, or the parental strain (parent) (7).

Similar to previous reports, e.c. exposure with the Δ proteases mutant resulted in markedly decreased skin inflammation compared to WT mice as determined by the disease score and epidermal thickening (Fig. 5 A–D). Importantly, mice e.c. exposed to the Δ proteases mutant had restoration of the skin barrier compared to mice e.c. exposed to the parent strain, as measured by significantly decreased TEWL (transepidermal water loss) measurements (Fig. 5E) and markedly increased transcript and protein expression of filaggrin (*Flg*/FLG) and desmoglein (*Dsg1*/DSG-1) (Fig. 5 F and G). Since we have previously shown that *S. aureus* induces IL-36 α expression in keratinocytes and IL-36R signaling is crucial for *S. aureus*–mediated skin inflammation (5, 26), we hypothesized that *S. aureus* proteases induced IL-36 α expression in the skin. Interestingly, the *S. aureus*–mediated induction of IL-36 α was not regulated transcriptionally, as mRNA expression was comparable between the *S. aureus* Δ proteases and parent strain–exposed skin (Fig. 5H). However, immunofluorescent staining for IL-36 α protein in the skin revealed significantly decreased IL-36 α production in the keratinocytes of

S. aureus Δ proteases-exposed skin compared to the parent strain (Fig. 5 I and J). Given these results, we hypothesized that *S. aureus* proteases mediated keratinocyte damage as a mechanism for the induction of IL-36 α in keratinocytes. Thus, we cocultured normal human keratinocytes with live or heat-killed parent and Δ proteases *S. aureus* strains and measured keratinocyte viability. We found that keratinocytes cultured with the Δ proteases strain had markedly increased viability compared to keratinocytes cultured with the parent strain (SI Appendix, Fig. S7). The loss of keratinocyte viability required live bacterial cells, as keratinocytes cultured with heat-killed *S. aureus* had comparable viability as control keratinocytes. Taken together, these results indicated that *S. aureus* proteases induced IL-36 α production in keratinocytes to drive skin inflammation, which was associated with decreased skin barrier integrity in vivo and reduced keratinocyte viability in vitro.

S. aureus Proteases Promote Recruitment of IL-17A-Expressing Eosinophils into the Skin.

Given *S. aureus* proteases induced IL-36 α production and IL-36R signaling is important for eosinophil

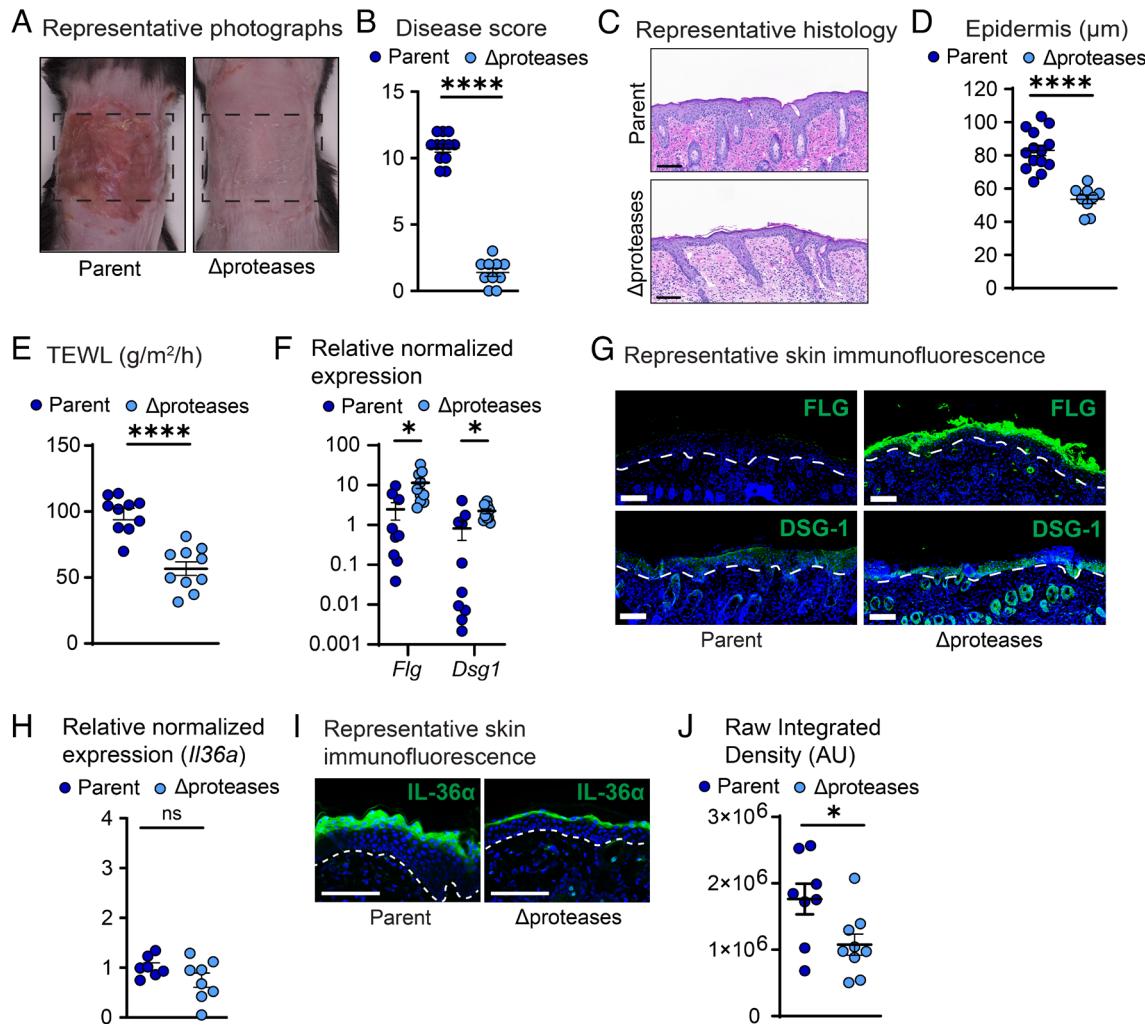


Fig. 5. *S. aureus* proteases degrade skin barrier integrity and induce IL-36 α production in keratinocytes. WT female mice were e.c. exposed to the parent or Δ proteases *S. aureus* strain (1×10^8 CFU) for 7 d, and a 10-mm skin biopsy was harvested ($n \geq 9$ per group). (A) Representative skin photographs. (B) Mean disease score (\pm SEM). (C) Representative histology (H&E stain, 200 \times magnification). (D) Mean epidermal thickness (\pm SEM). (E) TEWL of day 7 infected skin (\pm SEM). (F) Relative normalized expression of filaggrin (*Flg*) and desmoglein-1 (*Dsg1*), normalized to β -actin in parent strain–exposed skin (\pm SEM). (G) Representative immunofluorescence of filaggrin and desmoglein-1 (green) and DAPI (blue) at 400 \times magnification. The dotted lines represent the epidermal–dermal junction ($n \geq 4$ per group). (H) Relative normalized IL-36 α gene expression levels (\pm SEM), normalized to β -actin in parent strain–exposed skin ($n \geq 7$ per group). (I) Representative immunofluorescence for IL-36 α (green) and DAPI (blue) in the skin (400 \times magnification). The dotted lines represent the epidermal–dermal junction ($n \geq 8$ per group). (J) Quantification of epidermal IL-36 α expression levels from (F) using the raw integrated density measurement in ImageJ, normalized to the epidermal area ($n \geq 8$ per group). n.s.; not significant, * $P < 0.05$; *** $P < 0.0001$ as calculated by two-tailed Student's *t* test (B, D–F, H, and J). Scale bars are 100 μ m in (C, G, and I). Data are combined from at least two independent experiments.

trafficking, we lastly set out to determine whether *S. aureus* proteases contributed to the eosinophil responses in the inflamed skin. To this end, we first measured eosinophil levels in the skin by flow cytometry and found a significant reduction in eosinophils, but not T cells in the skin of mice exposed to the *S. aureus* Δproteases strain compared to the parent strain (Fig. 6A). Moreover, there was a significant reduction in *Ccl7* expression in the mice exposed to the *S. aureus* Δproteases strain compared to the parent strain, whereas expression of the other eosinophil-attracting chemokines was either increased or comparable between the strains (Fig. 6B). To determine whether *S. aureus* proteases also influenced CCL7 protein production, we performed immunofluorescent histology on day 7 skin and found a marked reduction in CCL7 in Δproteases e.c. exposed mice compared to mice e.c. exposed to the parent strain (Fig. 6C). Since eosinophils were a predominant source of

IL-17A and IL-17F in the inflamed skin, we reasoned that the *S. aureus* Δproteases strain would have decreased IL-17A and IL-17F expression in the skin. As expected, the parent *S. aureus* strain induced robust IL-17A and IL-17F expression compared to naive skin in the dual-color IL-17A-tdTomato/IL-17F-GFP reporter mouse, whereas the *S. aureus* Δproteases strain had significantly decreased IL-17A and IL-17F expression compared to the parent strain (Fig. 6D and E). These results were confirmed on the transcriptional level, as the skin from *S. aureus* Δproteases-exposed mice had significantly reduced expression of *il17a* and *il17f* as compared to the parent strain (Fig. 6F). Finally, our data revealed that mice challenged with the *S. aureus* Δproteases strain had markedly decreased IL-17A-expressing eosinophils and T cells in the skin compared to mice exposed to the parent strain (Fig. 6G). Collectively, these results suggested that *S. aureus* proteases initiated

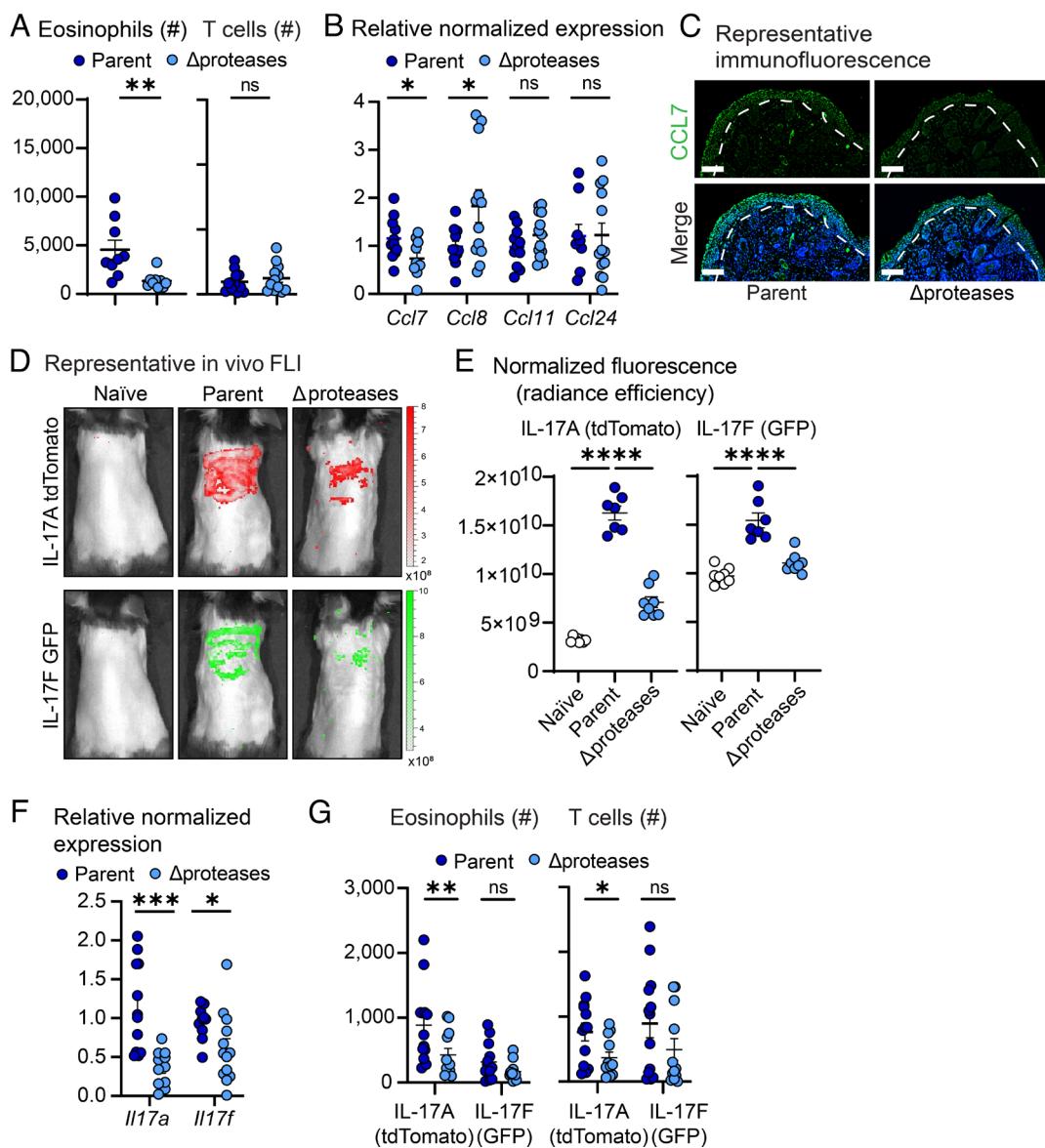


Fig. 6. *S. aureus* proteases trigger IL-17A-producing eosinophil recruitment to the skin inflammation. (A–C) WT female mice were e.c. exposed to the parent or Δproteases *S. aureus* strain (1×10^8 CFU) for 7 d, and a 10-mm skin biopsy was harvested ($n \geq 9$ per group). (A) Mean numbers of eosinophils and T cells (\pm SEM) in the skin as measured by FACS. (B) Relative normalized gene expression (\pm SEM) in the skin, normalized to β -actin in naive skin. (C) Representative skin immunofluorescence of CCL7 (green) and DAPI (blue) 400 \times magnification. The dotted lines represent the epidermal-dermal junction ($n = 4$ per group). (D–G) IL-17A-tdTomato/IL-17F-GFP dual reporter female mice were e.c. exposed to the parent or Δproteases *S. aureus* strain (1×10^8 CFU) on the dorsal skin for 7 d, and a 10-mm skin biopsy was harvested ($n \geq 7$ per group). (D) Representative in vivo FLI. (E) Mean total flux (photons/s) of IL-17A (tdTomato) and IL-17F (GFP) (\pm SEM). (F) Relative normalized gene expression levels (\pm SEM), normalized to β -actin in *S. aureus* parent-exposed skin. (G) Mean numbers of IL-17A+ (tdTomato) and IL-17F+ (GFP) eosinophils and T cells (\pm SEM) in the skin as measured by FACS. n.s.; not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ as calculated by two-tailed Student's *t* test (A, B, F, and G) or a one-way ANOVA multiple comparisons test with Tukey correction (E). Data are combined from at least two independent experiments.

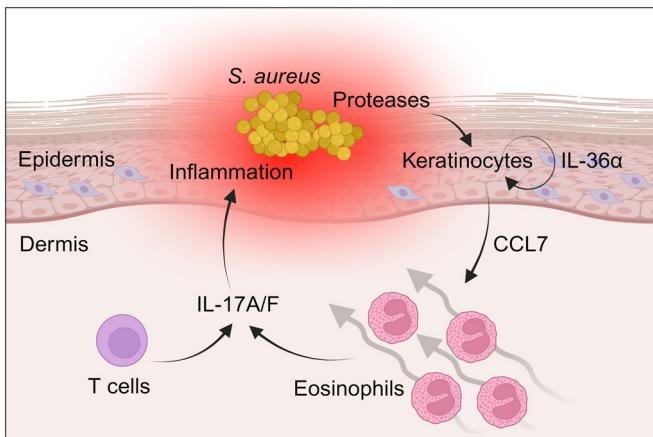


Fig. 7. Model of *S. aureus*-mediated skin inflammation. *S. aureus* proteases during e.c. exposure trigger IL-36 α autocrine signaling on keratinocytes, which promotes CCL7 production and recruitment of eosinophils. In combination with T cells, IL-17A- and IL-17F-producing eosinophils drive skin inflammation.

the recruitment of IL-17A-producing eosinophils into the skin during skin inflammation.

Discussion

S. aureus skin colonization and eosinophil infiltration are associated with many inflammatory skin disorders, including AD, BP, NS, and PN (3, 4, 7, 19–24). However, whether there is a relationship between *S. aureus* and eosinophils and how this interaction influences skin inflammation is largely undefined. Herein, we uncovered that *S. aureus* e.c. exposure induced eosinophil recruitment to the site of skin inflammation. Importantly, we found that eosinophils contributed to skin inflammation in a mechanism that involved eosinophil-derived IL-17A and IL-17F production. Furthermore, we found that *S. aureus* proteases induced IL-36 α production in keratinocytes, which drove CCL7 production and IL-17A- and IL-17F-producing eosinophil recruitment and skin inflammation (Fig. 7). Collectively, these findings provide important insights into eosinophil biology and inflammatory skin disease pathogenesis.

We found that eosinophils contributed to skin inflammation upon *S. aureus* e.c. exposure, which was associated with the increased expression of eosinophil-recruiting chemokines (e.g., *Ccl7*). Importantly, we uncovered that CCL7 contributes to *S. aureus*-mediated skin inflammation in a mechanism that involves eosinophil recruitment to the inflamed skin. Interestingly, *CCL7* expression is increased in various skin and allergic disorders (e.g., psoriasis, AD, rhinitis, and asthma), with circulating CCL7 protein levels in AD patients correlating with disease severity (16, 37–42). Furthermore, in a mouse model of allergic rhinitis (AR), CCL7-mediated eosinophil trafficking to the airways occurred upon nasal ovalbumin (OVA) administration (42). Interestingly, *Ccl8*, *Ccl11*, and *Ccl24* expression were not able to compensate for the reduced *Ccl7* in our model. This may be due to the high expression of CCL7 in keratinocytes in our model, whereas CCL11 is expressed in fibroblasts (43) and CCL24 is expressed in macrophages (44) during AD skin inflammation. Thus, differential cellular expression of eosinophil-recruiting chemokines may have important outcomes in the pathogenesis of *S. aureus*-mediated skin inflammation. Although our findings suggested that CCL7 is produced in keratinocytes, determining what cells produce CCL7 and the mechanistic role of CCL7 in human inflammatory skin diseases warrants further investigation. Our findings may be relevant to human disease, as AD patients colonized with

S. aureus have increased eosinophil counts in circulation compared to noncolonized patients (19). Our findings may also explain the clinical observation that circulating eosinophil levels correlate with disease severity in AD patients (19–21, 24, 25). However, clinical studies using the anti-IL-5 antibody, mepolizumab, and the anti-IL-5R antibody, benralizumab, in AD patients to target eosinophils did not show reductions in SCORAD, pruritus, or disease biomarkers compared to baseline (45, 46). In contrast, the anti-IL-5 antibody, reslizumab, showed efficacy in patients with BP (47, 48), and a clinical trial (FJORD study) is underway to test the use of benralizumab in BP patients (NCT04612790). Similar to AD, mepolizumab failed to treat BP patients, although this may be due to the lack of decreased eosinophils in the skin of treated patients (49). Thus, more studies are needed to determine the contribution of eosinophils in inflammatory skin diseases in humans.

Our findings revealed that eosinophils are a predominant source of IL-17A and IL-17F in the murine inflamed skin in response to e.c. *S. aureus* exposure, which was important for the development of skin inflammation in mice. Unexpectedly, eosinophils had a similar contribution to IL-17A and IL-17F production and skin inflammation as T cells, which were previously reported to be critical for *S. aureus*-induced skin inflammation (5, 6). We found that despite reduced disease scores in the absence of T cells, there was a compensatory increase in eosinophils in the skin. A possible explanation is that T cells and eosinophils have different niches in the skin to drive pathogenesis. Eosinophils store preformed cytokines in cytoplasmic granules (50) but additional studies are needed to determine whether IL-17A and IL-17F are preformed in granules or produced upon influx into the inflamed skin. Interestingly, we observed discordance in IL-17A- and IL-17F-expressing eosinophils, which may indicate distinct IL-17A and IL-17F eosinophil subsets in the skin. Our future work will unravel the role of eosinophil subsets in skin inflammation. Furthermore, our finding that eosinophils produced IL-17A and IL-17F implicates a broader role for eosinophils in the pathogenesis of other inflammatory diseases. For example, eosinophils promoted neutrophil recruitment and inflammation in mouse models of imiquimod-induced psoriasis-like dermatitis and *Aspergillus fumigatus* lung infection (51, 52). However, since we did not find a neutrophil recruitment defect in eosinophil-deficient mice but did observe a reduced neutrophil signature by RNA-seq analyses, our findings do not implicate a role for eosinophils in the recruitment of neutrophils but rather in regulation of neutrophil function. Notably, the anti-IL-17A antibody, secukinumab, was effective in a small cohort of NS patients (53), which corresponds to a recent study highlighting a significant IL-17 inflammatory signature in NS patient skin (30). Furthermore, IL-17 was shown to be important for BP pathogenesis in preclinical and in vitro models (32), with secukinumab successfully used to treat a patient with BP (54). Moreover, IL-17 response genes were enriched in PN patients compared to healthy controls (31). Last, the intrinsic, Asian, and pediatric endotypes of AD have increased Th17-related markers (e.g., IL-17A) compared to other AD endotypes (55), although secukinumab therapy was not effective in AD patients (56). Determining whether eosinophil-derived IL-17A and IL-17F contribute to inflammation in these human skin diseases warrants further investigation.

We identified that IL-36R signaling was critical for eosinophil recruitment to the inflamed skin, in a mechanism that involved increased *Ccl7* expression. This is likely relevant to human disease as we and others have shown that AD patients have increased IL-36 cytokine expression in circulation and in the lesional skin (26, 57), although the anti-IL-36R antibody, Spesolimab, had only modest efficacy in AD patients (58). A recent study found

that NS patients have a predominant IL-36 immune signature (30), with a clinical trial set to test Spesolimab in NS patients (NCT05856526). Moreover, IL-36 cytokines were overexpressed in BP and PN patient skin (31, 59). Similarly, IL-36 expression was associated with inflammation in other eosinophilic disorders (e.g., AR and eosinophilic pustular folliculitis) and induced the *in vitro* survival, adhesion, migration, and activation of eosinophils, suggesting that IL-36R signaling is an important mediator of eosinophilic responses (42, 60, 61). Regarding CCL7, Blumberg et al. found that transgenic mice with overexpression of IL-36 α in keratinocytes have increased CCL7 expression in the skin compared to nontransgenic mice (62), corroborating our results on the involvement of keratinocyte-specific IL-36R signaling on CCL7 expression in the skin. Whether IL-36R signaling also acts directly on eosinophils or indirectly on other cells in the skin and how this affects eosinophil responses to mediate disease pathogenesis will be the focus of future studies.

Finally, we found that *S. aureus* proteases drive skin inflammation in a mechanism that involves reduced skin barrier integrity, and induction of keratinocyte-derived IL-36 α and CCL7-mediated recruitment of IL-17A-producing eosinophils. Furthermore, we found that *S. aureus* proteases reduce human keratinocyte viability. A possible explanation for this phenotype includes protease-mediated cell death via protease-activated receptors (PARs) and NLRP1 activation, as previously reported (63–65). This is particularly relevant, as *S. aureus* skin colonization in AD patients increases NLRP1 inflammasome activation (66). Pathogen-derived proteases have been shown to directly induce and cleave IL-36 cytokines into their active forms (67), providing a possible explanation for the role of *S. aureus* proteases in the IL-36R-mediated skin inflammation. Another explanation is that since keratinocytes produce endogenous proteases upon *S. aureus* exposure (68), this results in IL-36 α activation and skin inflammation. *S. aureus* proteases may also directly interact with eosinophils, since eosinophils express PARs and are activated by environmental proteases (e.g., dust mites and fungi) (69). Further identifying the relationship between *S. aureus* proteases, IL-36 α activation, and eosinophils will be investigated in future work.

There were some limitations to our study. First, we did not identify the specific *S. aureus* proteases involved in our model. However, prior studies have shown the importance of the *S. aureus* cysteine proteases, staphopain A and B (e.g., scpA and sspB), in driving skin inflammation (8) and will be examined in future work. Additionally, we only tested a single strain of *S. aureus*, limiting the broader conclusions of our findings. Prior publications have found defects in basophils and other myeloid cells in the Eos $^{-/-}$ mouse used herein (27, 28). However, we did not observe any developmental defects in the myeloid cell compartment, except for eosinophil deficiency, which may reflect differences in tissues tested (e.g., skin), or the microbiomes between animal facilities (70–72). Moreover, we used eosinophils purified directly from the blood of IL-5tg mice for adoptive transfer experiments into Eos $^{-/-}$ mice, whereas another group used *in vitro* cultured eosinophils from the bone marrow of WT mice (73). We also had discrepancies between our flow cytometric and RNA-seq Xcell enrichment analyses. This may be explained by limitations in Xcell to infer cellular enrichment in complex tissue samples (74) or may suggest a change in function of the immune cells rather than in population levels. Also, our adoptive transfer and eosinophil depletion results were modest. However, a single injection of eosinophils on day 0 may not fully recapitulate the eosinophil influx in the WT mice during *S. aureus* exposure, which peaked on day 7. We also did not achieve complete depletion of eosinophils in the skin, which may have affected the outcome. The use of an alternative genetic model [e.g., PHIL mice (75)] would have further strengthened the study. Furthermore, our

results cannot make any conclusions on sex-based differences, as we focused our studies on female mice. Last, we focused on the role of eosinophil-derived IL-17A and IL-17F, despite our RNA-seq KEGG pathway analysis revealing various immune pathways associated with eosinophils in the inflamed skin (e.g., JAK/STAT signaling and Th1 and Th2 cell differentiation) (29, 76). Understanding how these pathways are involved in eosinophil-mediated skin inflammation warrants further investigation.

In conclusion, we uncovered that *S. aureus* e.c. exposure induces eosinophil recruitment to the lesional skin in a mouse model of skin inflammation. Eosinophils contributed to skin inflammation in a mechanism that involved endogenous IL-17A and IL-17F production and upstream IL-36R signaling. Importantly, we revealed that *S. aureus* proteases triggered IL-36 α expression in keratinocytes to drive CCL7 production and downstream IL-17A-producing eosinophil recruitment and skin inflammation. This pathway provides potential therapeutic targets for the treatment of inflammatory skin disorders.

Materials and Methods

Original data created for the study are or will be available in a persistent repository upon publication.

Experimental Model and Subject Details.

S. aureus strains. The bioluminescent *S. aureus* LAC4303 strain (also designated SAP430) was generated from *S. aureus* strain JE2 (BEI Resources), which is the LAC strain but cured of its native plasmids (77), and by integrating plasmid pPR1195 into the chromosome as previously described (78). This bacterial strain also possesses a modified *lux* operon from *Photorhabdus luminescens*, stably integrated into the bacterial chromosome so that the emission of blue-green light from live and metabolically active bacteria is maintained in all progeny without selection. The 10-protease-deficient (AH1919) and parent (AH1263) *S. aureus* strains were a generous gift from Alexander Horswill (University of Colorado) (79).

Bacterial preparation. *S. aureus* USA300 LAC4303 bacteria were streaked onto a tryptic soy agar (TSA) plate [tryptic soy broth (TSB) plus 1.5% bacto agar; BD Biosciences] and grown overnight at 37 °C. Three single colonies were picked and cultured in TSB at 37 °C in a shaking incubator (240 rpm) overnight (18 h), followed by a 1:50 subculture at 37 °C for 2 h to obtain mid-logarithmic growth phase bacteria. Bacteria were then pelleted, washed, and resuspended in sterile PBS, and the absorbance at 600 nm (A_{600}) was measured to estimate the CFU (1×10^8 CFU/100 µL) (5, 6, 26), which was verified by overnight culture on TSA plates.

Mice. Gender- and age-matched 6- to 9-wk-old mice on either a C57BL/6 or BALB/c background were used for all experiments. WT mice on a C57BL/6 background, WT on a BALB/c background, Rag1 $^{-/-}$, and C.129S1(B6)-Gata1 tm16sh (Eos $^{-/-}$) mice on a BALB/c background were purchased from Jackson Laboratories (Bar Harbor, ME). Eos \times Rag1 $^{-/-}$ mice on a BALB/c background were generated as an in-house cross between Eos $^{-/-}$ and Rag1 $^{-/-}$ mice. The following mice were all on the C57BL/6 background: IL-17A-tdTomato/IL-17F-GFP dual-color reporter mice were provided by Scott Durum (NIH) and generated as previously described (63). IL-36R $^{-/-}$ mice were generated in-house as previously described (26). K14 cre -IL-36R $^{fl/fl}$ mice were generated as an in-house cross between K14-cre mice (Jackson Labs #018964) and IL-36R $^{fl/fl}$ mice (26). This generated K14-cre \times IL-36R $^{fl/fl}$ mice on a C57BL/6 background, which lack IL-36R-signaling on K14-expressing cells (e.g., keratinocytes). IL-17A/F $^{-/-}$ mice were provided by Yoichiro Iwakura (University of Tokyo, Japan) and generated as previously described (80). Interleukin-5 transgenic mice (IL-5tg) on a C57BL/6 background were provided by Elizabeth A. Jacobsen (Mayo Clinic, MN) and generated as previously described (81).

Study approval. All mouse strains were bred and maintained under the same specific pathogen-free conditions, with air-isolated cages at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at Johns Hopkins University and handled according to procedures described in the Guide for the Care and Use of Laboratory Animals as well as Johns Hopkins University's policies and procedures as set forth in the Johns Hopkins University Animal Care and Use Training Manual. All animal experiments were approved by the Johns Hopkins University Animal Care and Use Committee. The collection and use of

primary human keratinocytes was approved by the Johns Hopkins School of Medicine Institutional Review Board (#00031269).

Mouse model of *S. aureus* e.c. exposure. A mouse model of e.c. *S. aureus* exposure was used as previously described (5, 6, 26, 82). In brief, the dorsal skin of anesthetized mice (2% isoflurane) was shaved and depilated (*Nair* cream) 1 d prior to bacterial inoculation. A 100 μ L volume of PBS containing 1×10^8 CFU of USA300 LAC4303 was placed on a sterile gauze pad (1 \times 2 cm) and attached to the shaved dorsal skin with transparent bio-occlusive dressing (Tegaderm; 3M), followed by two layers of adhesive bandages (BAND-AID, Johnson and Johnson) for 7 d. The disease score as a measure of skin inflammation severity was determined by a blinded observer from digital photographs and was quantified using a disease score: the sum of three individual grades for erythema (graded: 0, 1, 2, 3), edema (graded: 0, 1, 2, 3), and scaling/erosion (scaling graded: 0, 1, 2, 3 or erosion graded: 4, 5, 6, 7), for a total range of 0 to 13 (*SI Appendix, Fig. S8*) (26).

Quantification and Statistical Analysis Data from single comparisons were analyzed by a two-tailed Student's t test. Data from more than two comparisons were analyzed by a one-way ANOVA multiple comparisons test with Tukey or Dunnett correction, as indicated in the figure legends. All statistical analyses were performed using Prism software version 9 (GraphPad). Data are presented as mean \pm SEM and values of $P < 0.05$ were considered statistically significant.

Data, Materials, and Software Availability. Raw and processed RNA-seq data have been deposited at NCBI's GEO database under accession number [GSE216246](#) and will be publicly available as of the date of publication (83). All other data are included in the manuscript and/or *SI Appendix*.

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