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Staphylococcus aureus and *S. epidermidis* strain diversity underlying human atopic dermatitis

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Abstract

The heterogeneous course, severity, and treatment responses among patients with atopic dermatitis (AD; eczema) highlight the complexity of this multifactorial disease. Prior studies have used traditional typing methods on cultivated isolates or sequenced a bacterial marker gene to study the skin microbial communities of AD patients. Shotgun metagenomic sequence analysis provides much greater resolution, elucidating multiple levels of microbial community assembly ranging from kingdom to species and strain-level diversification. Here, we analyze microbial temporal dynamics from a cohort of pediatric AD patients sampled throughout the disease course. Species-level investigation of AD flares showed greater *Staphylococcus aureus*-predominance in patients with more severe disease and *S. epidermidis*-predominance in patients with less severe disease. At the strain-level, metagenomic sequencing analyses demonstrated clonal *S. aureus* strains in more severe patients and heterogeneous *S. epidermidis* strain communities in all patients. To investigate strain-level biological effects of *S. aureus*, we topically colonized mice with strains isolated from

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Data and materials availability: The sequencing data for this study are linked to NCBI Bioproject 46333.

Supplementary materials:
Materials and Methods
Table S1–S10
Figures S1–12

AD patients and controls. This cutaneous colonization model demonstrated *S. aureus* strain-specific differences in eliciting skin inflammation and immune signatures characteristic of AD patients. Specifically, *S. aureus* isolates from AD patients with more severe flares induced epidermal thickening and expansion of cutaneous Th2 and Th17 cells. Integrating high-resolution sequencing, culturing, and animal models demonstrated how functional differences of staphylococcal strains may contribute to the complexity of AD disease.

Atopic dermatitis (AD, eczema) is a common inflammatory skin disorder in industrialized countries, affecting 10–30% of children (1). Patients with AD suffer from chronic, relapsing, intensely itchy, inflamed skin lesions, and have an increased likelihood of developing asthma and/or hay fever (2). AD is a complex multifactorial disease in which epidermal barrier impairment, type 2 immunity, and skin microbes are each thought to play a causative role (1). Over 30 susceptibility loci have been associated with AD, including mutations in the gene encoding the skin barrier protein filaggrin (*FLG*) (3) and genes linked to the immune system (4).

In addition to host genetic susceptibility, the relationship between AD and skin bacteria is well-recognized clinically. Patients with AD are often treated with varying combinations of antimicrobial approaches (e.g. antibiotics and dilute bleach baths) and anti-inflammatory or immunosuppressive medications (5). The efficacy of these antimicrobial treatments is associated with decreases in staphylococcal relative abundances (6, 7). *Staphylococcus aureus* commonly colonizes AD skin and has been studied using colony-counting, sequence typing methods (e.g. pulsed field gel electrophoresis, SpA typing) of selected cultivated isolates (8–11), and more recently, amplicon-based (marker gene) sequencing of the 16S rRNA gene (6, 7, 12, 13). However, sequence typing and amplicon sequencing methods are unable to distinguish between genetically distinct strains as determined by whole genome sequencing (14, 15). By contrast, shotgun metagenomic sequencing of skin samples from healthy individuals provided deeper resolution and demonstrated the multiphyletic composition of commensal *Staphylococcus* (16).

With an increasing appreciation of functional differences between strains within a single species, we performed shotgun metagenomic sequencing of AD patient skin samples to capture the full genetic potential and strain-level differences of the skin microbiome throughout the course of disease. We confirmed an increase of staphylococcal species during disease flares in our cohort and more deeply explored the *S. aureus* and *S. epidermidis* strain diversity of each patient. To test the functional consequence of strain-level differences between patients, we isolated staphylococcal strains from patients and healthy controls and investigated the cutaneous and immunologic effects when applied topically in a mouse model.

Bacterial communities shift during AD disease progression

To examine the relationship between the skin microbiota and AD, eleven children with moderate-to-severe AD and seven healthy children were recruited to the NIH Clinical Center between June 2012 and March 2015 (Table S1,S2). As AD has a chronic relapsing course, patients were sampled at stable/typical disease state (baseline/B), worsening of disease

(flare/F) and 10–14 days after initiation of treatment using a combination of skin-directed therapies (post-flare/PF). Since the use of topical medications on AD skin alters skin bacterial communities (6, 7), baseline samples were defined as those collected from subjects in their routine disease state who refrained from using skin-directed antimicrobial and anti-inflammatory treatments for seven days, a duration of time determined based on prior findings (6). Flares were defined as timepoints when patients experienced worsening in the clinical severity of their typical AD, had not used skin-directed antimicrobial and anti-inflammatory treatments for seven days, and did not have clinical skin infection (i.e. yellow crusts or pustules). At each timepoint, disease severity was determined with objective SCORAD (SCORing Atopic Dermatitis), a validated clinical severity assessment tool (17–19). Subjects were sampled bilaterally at sites of disease predilection, the inner elbow (antecubital crease/Ac) and behind the knees (popliteal crease/Pc), along with five additional sites to investigate defined areas with different skin physiologies (fig. S1). Due to the clinical severity of their AD, six of the eleven patients experienced exacerbations of their skin disease with the seven-day skin preparation regimen and could not provide baseline timepoint samples, reflecting the spectrum of the natural course of AD. Because the skin microbial dysbiosis during AD flares was of greatest interest, the majority of the analyses focused on comparisons between flare and post-flare timepoints. In total, we performed shotgun metagenomic sequencing of 422 samples, generating 191 Gb of microbial sequence data from 27 AD patient visits and 7 healthy control visits (Table S3). During patient flares, AD disease severity was significantly elevated as indicated by higher mean objective SCORAD (38 ± 2.9) as compared to baseline (9.4 ± 1.6, $p < 4.5\times10^{-4}$) and post-flare (11 ± 1.6, $p < 2.8\times10^{-6}$) (Fig. 1A).

To compare the microbial community composition across timepoints, we mapped microbial reads to a multi-kingdom reference database. As seen in healthy adults (16, 20, 21), bacteria was the most predominant kingdom across timepoints and body sites (fig. S2, Table S4), *Malassezia* species, particularly *M. restricta* and *M. globosa*, predominated the fungal communities (fig. S3, Table S5), and eukaryotic DNA viral communities were mostly polyomaviruses or papillomaviruses depending on the individual (fig. S4). No significant differences in the fungal or viral components over time were identified; therefore, we focused on bacterial communities that demonstrated the greatest shifts in this cohort (fig. S5, Table S6). We first determined the Shannon diversity index, an ecological measure of richness (total number of bacterial species) and evenness (relative proportion of the bacterial species), to evaluate the overall community structure/composition across body sites and timepoints. During flares, sites of AD predilection (Ac and Pc) exhibited a marked reduction in Shannon diversity compared to baseline, post-flare, and healthy controls, a trend observed to a lesser extent across other sites (Fig. 1B). Since changes in bacterial diversity were most pronounced at the sites of disease predilection and Ac/Pc have similar microbial communities (21), we averaged these sites per subject and used the composite “AcPc” for subsequent analyses. Similar to our previous analysis of microbial diversity in an AD patient cohort (6), the partial correlation between objective SCORAD and Shannon diversity, adjusting for disease state, was significantly inversely correlated ($r = -0.58$, $p = 4.5\times10^{-4}$) (Fig. 1C), indicating that reduced skin bacterial diversity corresponds to worse disease severity, primarily at sites of disease predilection (fig. S5A).

To determine which taxa were contributing to the loss of diversity, we compared the relative abundances of the most prominent taxa (Fig. 1D and fig. S5B). Of the four most prominent genera in the AcPc, only *Staphylococcus* was significantly increased in flares (45 ± 10.2%) as compared to post-flares (9.2 ± 2.4%, $p < 0.0078$) and healthy controls (6.6 ± 4.1%, $p < 0.033$) (Fig. 1E). This increase in *Staphylococcus* relative abundances was positively correlated with objective SCORAD (r=0.67, $p < 8.1\times10^{-6}$)(Fig. 1F), indicating that severe AD was associated with higher staphylococcal relative abundances at sites of disease predilection. In addition, there was a positive correlation for the forehead, retroauricular crease, and volar forearm (fig. S5C), sites that can be affected in more severe disease. However, differences in *Corynebacterium*, *Propionibacterium*, and *Streptococcus* relative abundances between flares and post-flares were not statistically significant (Fig. 1E).

AD flare severity associated with specific staphylococcal species

To further examine the positive correlation between *Staphylococcus* and AD disease course (22), we identified the relative abundances of staphylococcal species including *S. aureus, S. epidermidis, S. hominis*, and *S. capitis* (Fig. 2A and fig. S6). Only relative abundances of *S. aureus* were significantly increased from flares (28 ± 8.8%) to post-flares (2.3 ± 0.8%, $p < 0.014$)(Fig. 2B). While *S. epidermidis* relative abundances were also higher during flares (13 ± 5.4%) as compared to post-flares (3.7 ± 1.4%), results did not reach statistical significance. For all patients, relative abundances of *S. aureus* were positively correlated with objective SCORAD (r = 0.73, $p < 1.\times10^{-7}$), while *S. epidermidis* was not correlated (Fig. 2C and fig. S7). This association between *S.aureus* and AD severity (23) has been observed in prior studies. Neither *S. hominis* nor *S. capitis* demonstrated significant shifts in relative abundances between timepoints (Fig. 2B) or were correlated with disease severity (fig. S7).

To explore further the relationship between disease severity and staphylococcal species, we sorted the patients by their objective SCORAD and plotted the relative abundances of *S. aureus* and S. *epidermidis* at flare (Fig. 2D). We observed a trend whereby patients with more severe AD flares (objective SCORAD 45 ± 3.0) had higher relative abundances of *S. aureus* (Fig 2D bottom row, fig. S8, and Table S7). In contrast, patients with less severe AD flares, and lower objective SCORAD (31 ± 1.9, $p < 0.004$ in comparison to the more severe flares), had higher relative abundances of *S. epidermidis* (Fig. 2D top row) across sampled sites. Specifically, more severe AD flare patients had relative abundances of 34 ± 8.7% *S. aureus* with 7.4 ± 4.2% *S. epidermidis* and less severe AD flare patients had relative abundances of 3.8 ± 1.7% *S. aureus* with 13 ± 3.9% *S. epidermidis* averaged across all sites during flare. The range of *S. aureus* relative abundances based upon sequencing was variable: 3 of 11 patients had no *S. aureus* on their skin and 3 of 11 had relative abundances of *S.aureus* on their skin exceeding 50%, similar to prior studies of *S. aureus* relative abundances on AD skin.

To compare these metagenomic results with more traditional studies, we cultured bacteria from skin and nares swabs collected concurrently with genomic samples. Cultures of *S. aureus* from skin clinical samples correlated with the microorganism detection by sequencing. Of note, two less severe AD flare patients were culture-positive for *S. aureus*

only in their nares, a common site of carriage. The *S. aureus* culture-positive rates in this cohort was consistent with other studies (6–8, 11–13). The genomic analyses were internally consistent with cultivation results and both supported the strong association between AD disease severity and *S. aureus*.

Monoclonal *S. aureus* strains observed in AD

While the differential association of *S. aureus* and *S. epidermidis* with AD severity defined an intriguing feature of disease heterogeneity, the underlying strain communities of these species during the disease course remained unknown. Two alternative scenarios could underlie microbial shifts in a disease flare: a) all strains equally increase in relative abundance or b) particular strain(s) bloom and drive the increase. This distinction is important as individual strains may exhibit functional differences. In previous studies, this question could not be addressed, as traditional typing and amplicon-based sequencing methods may differentiate clonal complexes but miss gene content and single nucleotide variant (SNV) variants (14, 15). In contrast, shotgun metagenomics provides resolution of microbial communities at a strain and SNV level (24). We used our previously validated strain-tracking approach to identify strains of *S. aureus* and *S. epidermidis* present on our AD patients (16, 21). For *S. aureus* strain-tracking, microbial reads were mapped against a database composed of 215 *S. aureus* genomes, of which 61 representatives are shown in Figure 3A.

In contrast to the heterogenous communities of *P. acnes* and *S. epidermidis* strains observed in healthy adult skin (16, 21), the more severe AD patients were strikingly colonized with a single clade of *S. aureus* during disease flares (patients exhibited no clinical signs of infection) (Fig. 3B, fig. S9, and Table S8). In 4 of the 5 severe AD flare patients, this colonization with a single strain persisted in the post-flare but at notably lower mean relative abundances. AD patient AD11 was the exception, colonized by 3 different clades of *S. aureus* (E17, E7, and B1) with only clade E17 predominating during a flare. Notably, these more severe AD patients were colonized with distinct *S. aureus* clades. This supports previous studies demonstrating AD patients do not share a single dominant *S. aureus* clone (11, 25–27). The variation in the clonal *S. aureus*-clades colonizing AD patients raises the possibility that this heterogeneity may contribute to the differential course and/or therapeutic responses of AD patients.

To confirm our strain-tracking results, we used a complementary approach in which SNVs were identified in the *S. aureus* core genome (1.9 Mbps shared between all sequenced *S. aureus*). To power this analysis, we combined all sites and timepoints for each patient. In total, we identified 38,867 variant positions in the *S. aureus* core or ~10,000 SNPs per patient. We then used the degree of polyallelism in each individual to infer genetic heterogeneity or the presence of multiple *S. aureus* strains. We calculated the number of mono, bi, and triallelic SNVs for each patient (Fig. 3C). Consistent with strain-tracking results, SNVs in clonal *S. aureus*-colonized AD patients were monoallelic at 93% of sites, while heterogeneous patient AD11's SNVs were monoallelic at only 53% of sites.

S. aureus isolates cultured from each of the more severe AD flare patients underwent whole genome sequencing to confirm that the cultured patient isolates grouped into the respective clades predicted by strain-tracking of the metagenomic data (Fig. 3A). Colony-picking from cultured swabs for patient AD11 isolated a representative from the dominant E17 and the non-dominant B1 clade. Based on standard sensitivity-testing methods and whole genome sequencing analysis, five of the six *S. aureus* isolates from the more severe AD patients were methicillin-sensitive *S. aureus* (MSSA), consistent with higher incidences of MSSA than methicillin-resistant *S. aureus* (MRSA) cultivated from AD patient skin (28–30).

Comparative genomic analysis of these six *S. aureus* strains revealed extensive heterogeneity in the gene content as predicted based on the initial mapping of the shotgun metagenomics sequences to disparate phylogenetic clades. The genome of a single *S. aureus* isolate encodes̃2,500 genes of which ~85% (2128 genes) are present in every strain's genome and constitute the functional core (Fig. 3D); the remaining ~300 genes derive from the flexible pangenome comprised of 1,020 genes. We looked for functional enrichment in noncore versus core genes to identify pathways that were the most variable between our isolates (Table S9). In doing so, we identified the KEGG pathways ko05150 *Staphylococcus aureus* infection, ko00906 Carotenoid biosynthesis, and ko01501 beta-Lactam resistance as functionally enriched in the variable component of the pangenome. With a targeted search, enterotoxin genes, previously shown to exacerbate AD (31), were differentially present in the 6 AD patient strains of *S. aureus*. The 5 genes in the carotenoid biosynthesis pathway were present in all genomes but AD01.F1; this isolate is most closely related to strain MSHR1132 that was recently reclassified as *Staphylococcus argenteus* and can be visually distinguished by its white versus yellow pigment (32). Finally, variability of genes in the beta-Lactam resistance family, including the mec cassette, was consistent with our previous result that only isolate AD11.E17 was an MRSA. Overall, this strain-level gene variation generates additional questions regarding the potential role of specific strains on disease pathogenesis and host factors on clonal strain selection.

Heterogeneous *S. epidermidis* strain communities

To further address the microbial community structure, we explored whether AD patients harbored heterogenous communities of *S. epidermidis* on skin. For S. *epidermidis* strain-tracking, microbial reads were mapped against a database composed of 61 sequenced, phylogenetically diverse *S. epidermidis* genomes (Fig. 4A). As seen with healthy adults (21) and children, AD patients' *S. epidermidis* communities at both flares and post-flares were composed of multiple different strains from diverse clades of the phylogenetic tree (Fig. 4B. fig. S10, and Table S10). This directly contrasts with the identification of clonal *S. aureus* communities. This heterogenous *S. epidermidis* strain diversity was observed for both the more severe and less severe AD flare patients (fig. S10). However, analysis of the *S. epidermidis* strain composition in this cohort and our previous cohort of healthy adults (21) revealed a clustering of the less severe AD flare patients (Fig. 4C). Specifically, unsupervised clustering and principal coordinate analyses both identified *S. epidermidis* clades A29 and A30 as contributing to the clustering of the less severe AD patients and clade A20 as contributing to the clustering of the healthy adults (Fig. 4D). In contrast, the *S.*

epidermidis strain diversity in healthy control children and more severe flare patients were intermixed.

S. epidermidis clades A29 and A30 were enriched in strains originally collected from nosocomial infections rather than as skin commensals (33)(indicated with *s in Fig. 4A). Comparative genomic analysis of nosocomial isolates and the other strains revealed higher relative abundances of the SCC*mec* cassette (33), which encodes genes necessary for methicillin-resistance, in the nosocomial isolates. To further evaluate the *S. epidermidis* strains in this cohort, isolates were cultured from swabs collected from less severe patients AD05 and AD10. Whole genome sequencing confirmed the patient isolates as members of the A29 and A30 clade, respectively (Fig. 4A in red). Consistent with the trend of increased antibiotic resistance genes observed through genomic analysis, these patient isolates were methicillin-resistant *S. epidermidis*. A potential explanation for the overrepresentation of isolates genomically similarto nosocomial strains in less severe AD flare patients may be that these *S. epidermidis* strains outcompete commensals and/or *S. aureus* in inflammatory or non-steady-state conditions or that antibiotic usage in these patients may have selected for antibiotic resistance genes.

Strain-specific differences in cutaneous immune response in a murine model

While *S. aureus* has been tightly linked with AD, it is still debated whether *S. aureus* is a cause or effect, *i.e.* whether *S. aureus* can elicit and/or worsen AD skin disease or is a bystander that flourishes with increased access to extracellular matrix or other products of inflammation in eczematous skin (34, 35). Observing that individual strains of *S. aureus* predominated during AD flares in our more severe flare patients, we sought to investigate if these clonal strains elicited a biological response distinct from other strains of staphylococci. Harnessing the combined power of shotgun metagenomic sequencing of clinical samples and whole genome sequencing of bacteria cultivated from concurrently collected skin swabs, we next analyzed a) if strains associated with AD flares would be sufficient to elicit skin inflammation in the absence of any known genetic predisposition or prior barrier disruption and b) if there were strain-specific differences. To test this, we topically applied staphylococcal strains cultivated from AD patients and healthy controls onto intact skin of C57BL/6 wild-type mice with a method previously developed to test the immune response to skin commensals (Fig. 5A) We individually tested 10 phylogenetically distinct *S. aureus* isolates: six cultivated directly from the flared skin of patients with more severe flares, two from the skin of less severe patient AD07's flare timepoint, one *S. aureus* from a healthy control, and a common pathogenic *S. aureus* USA300 FPR3757 isolate (highlighted in red in fig. S9A). In addition, we tested three *S. epidermidis* isolates from AD patients: a representative from the clades A29 and A30 which predominated in the skin of less severe AD patients, and a representative from the ubiquitous B clade (highlighted in red in fig. S10A). In contrast to the non-inflammatory responses observed following association with either skin commensals (36, 37) or AD patient *S. epidermidis* isolates, topical application of the *S. aureus* isolates, particularly those associated with more severe AD flare patients, was sufficient to induce epidermal thickening and inflammatory responses (Fig. 5B,C, fig. S11A)

as well as immune cell infiltrate composed of neutrophils and eosinophils (Fig. 5D, fig. S11B,C). Interestingly, the USA300 isolate, commonly used as a representative *S. aureus* in functional experiments, induced only a modest immune response as compared to many of the isolates cultivated from severe AD patients, underscoring the importance of utilizing matched clinical isolates.

In addition to innate immune cells, infiltration of T cell receptor (TCR) $\alpha\beta^+$ and $\gamma\delta^{low}$ cells were also observed (fig. S12A) in mice colonized only with specific *S. aureus* strains. The majority of TCRβ^+ cells were CD4$^+$ (Fig. 5E) with variable effector potential, depending on the associated isolate. Notably, four *S. aureus* isolates from more severe AD flare patients induced production of the cytokine interleukin-13 (IL-13) (Fig. 5F), which is commonly associated with allergic inflammation. Cutaneous Th17 cells were also identified when mice were colonized with the four IL-13 inducing strains, in addition to AD07.B2 and USA300 (Fig. 5G). Recent reports have identified the presence of Th17 cells in AD lesions (38, 39), particularly in the Asian patient populations (40). Similar to CD4$^+$ T cells, the $\gamma\delta$ T cells of mice associated with specific strains of *S. aureus* isolates also had the potential to make higher levels of interleukin-17A (IL-17A) (fig. S12B). Notably, four of the *S. aureus* isolates [2 from more severe flare patient AD11 (AD11.B1, AD11.E17), 1 from less severe flare patient AD07 (AD07.E7), and the isolate from a healthy child (HC.B1)] induced minimal immune responses in all categories. Overall, association of *S. aureus* strains isolated from more severe AD flare patients to wild-type mice without prior barrier disruption induced immune responses in the skin that were significantly greater than those induced with *S. epidermidis* or *S. aureus* isolates from less severe AD flare patients or controls. Thus, these findings suggest specific strains of *S. aureus* may be sufficient to elicit and/or exacerbate skin inflammation as part of AD disease pathogenesis.

Discussion

AD is a complex disease with many contributing factors including skin barrier integrity, innate and adaptive immunity, and the microbiome. The heterogeneity of the course, severity, and clinical response in AD patients underscores the diversity of phenotypic presentations, as well as the probable differences in disease pathogenesis, within this one diagnosis. In addition to the various genetic susceptibility loci for AD, deeper investigation into the skin microbiome could provide better understanding of the microbial heterogeneity of AD and its potential contributions to disease.

While there have been many efforts to identify bacteria in AD skin, studies have generally relied on methods which do not distinguish microbes beyond the species-level or can misclassify genomically distinct clones (14, 15). Here, we combined shotgun metagenomic sequencing of clinical samples with whole genome sequencing of patient-derived isolates to investigate the microbial communities of AD skin down to the strain and SNV-level resolution. Since topical anti-inflammatory and anti-microbial treatments alter the skin microbiota (6, 7), the baseline and flare timepoints in this cohort were strictly defined by skin preparatory regimens to capture the natural history of the skin disease and to avoid potential confounders. As compared to healthy controls, the AD patients exhibited striking skin bacterial dysbiosis during flares. This dysbiosis was related to the increased relative

abundance of staphylococci, consistent with prior cohorts. Based on the disease severity (defined by objective SCORAD) during flares, we observed a strong correlation between severe AD flares and *S. aureus* relative abundances. These findings demonstrated that despite the relatively small numbers of subjects in this study, our cohort of patients is representative of other published patient cohorts as defined by validated diagnostic criteria.

Shotgun metagenomic sequencing enables strain-level examination of microbes within the broad microbial community of bacteria, fungi, and viruses. Strain-tracking identified striking outgrowth of clonal *S. aureus* strains in the skin of flaring AD patients with more severe disease; these same strains persisted post-flare at lower relative abundances. Other methods have examined whether *S. aureus* expansion in the skin of AD flares was related to either proportional increases in the entire community of *S. aureus* strains or the increase of a single or a few dominant clones; however, these studies were limited by the inability to examine these possibilities in the context of the whole skin microbial community. While the fungal and viral communities were not significantly different in this study, expansion of reference databases/genomes and studies into the microbial ‘dark matter’ in metagenomic data may provide further insights into AD microbiota. Our findings demonstrate that AD skin flares in patients with more severe disease are tightly linked with clonal *S. aureus* isolates.

In addition to characterizing strain communities during the course of AD, we found that less severe AD patients were colonized with more methicillin-resistant strains while the more severe AD patients were primarily colonized with methicillin-sensitive strains. While methicillin resistance is not as common in AD as would be predicted based on the high rates of *S. aureus* colonization in this disease, the finding of MSSA and MRSE predominance may contribute to differential responses to therapies in AD patients (41). The contrasts between *S. aureus* and *S. epidermidis* observed in this study likely also relate to the differences in microbial genetics and population dynamics at both the species and strain levels. Additional investigations of these microbiome phenotypic differences may improve the understanding of AD pathogenesis and lead to more targeted therapeutics. Birth cohort studies may address whether these patients acquired bacterial strains from family members and/or environmental sources as part of microbial inheritance (42). Testing of *S. aureus* strains in gnotobiotic mice, similar to *Bacteroides* gut commensal studies, may functionally address whether colonization by clonal *S. aureus* occurs through limited exposure or colonization resistance (43).

Using strains isolated from the skin of AD flares and a healthy control as well as a known laboratory strain, we examined the potential biological differences between staphylococcal strains. In a murine model without prior skin barrier disruption and with intact immunity, *S. aureus* strains from flare timepoints in more severe AD patients were sufficient to induce manifestations of skin inflammation, such as epidermal thickening and cutaneous infiltration of Th2 and Th17 cells. The magnitude of different immunologic effects varied depending on the isolated strain but was not strictly related to the disease severity of the source patient. Notably, murine colonization with either isolate AD11.B1 or AD11.E17 induced minimal immune responses even though patient AD11 has an objective SCORAD of 51.4. However, AD11 is also heterozygous for a null mutation in the FLG gene (S757X), suggesting that AD11’s strains of *S. aureus* may be immunogenic in the setting of an impaired skin barrier,

which previous studies have shown allows *S. aureus* to breach the epidermis into the dermis where it can trigger expression of proinflammatory cytokines (44). Caveats of these findings in the murine model are the relatively small number of isolates from this cohort that were fully sequenced and studied in the murine model and the observation of varied host responses when testing isolates from the same clade (AD04 and AD11), highlighting the need to examine a larger number of isolates including strains from similar and different clades and from healthy individuals and AD patients. An important additional limitation is the recognition that this murine model as well as others do not recapitulate the multiple complexities of human AD.

In mouse models, *S. aureus* enterotoxins have been shown to act as superantigens that can initiate Th17 responses (45), while *S. aureus* δ-toxin can induce degranulation of mast cells (46). These genes were both present in the non-inflammatory *S. aureus* isolates indicating strain-variability exists not only in gene content but also gene expression. Since healthy control-associated *S. aureus* strains were limited in our cohort due to the small percentage of healthy individuals colonized with *S. aureus*, future studies with additional *S. aureus* isolates from healthy individuals are necessary to tease apart the mechanisms underlying functional differences between *S. aureus* strains. In the context of prior studies demonstrating cutaneous immunologic responses to skin commensals (36, 37) and exacerbation of eczematous skin in AD mouse models by *S. aureus* (36, 37, 46, 47), our findings demonstrate that staphylococcal strains may play an important role in AD disease progression in a strain-specific manner.

In this study, we used shotgun metagenomic sequencing to examine strain-level microbial compositions of AD skin coupled with whole genome sequencing of patient isolates. With increasing recognition of highly individualized skin microbiomes (16), the presence of patient-specific strains underscores the individuality of the disease course and therapeutic response and may represent an opportunity for precision medicine. Our functional studies with cutaneous colonization of AD patient-associated strains of *S. aureus* and *S. epidermidis* demonstrated strain-specific differences in the ability to elicit histologic and immunologic alterations. AD typically has an age of onset in the first year of life when the human immune system is developing and being tuned by the endogenous microbial community. Recent studies have shown that early exposures can modulate host immunity to subsequent exposure and induce tolerance (48, 49). Thus, in light of the known links between severe AD and subsequent development of asthma and hay fever (“the atopic march”), targeted modulation of an AD patient’s particular staphylococcal strains has the potential to ameliorate the broader development of atopic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One Sentence Summary

Genomic and functional analyses of staphylococcal strain specificity reveal roles for microbes in human atopic dermatitis skin pathogenesis.

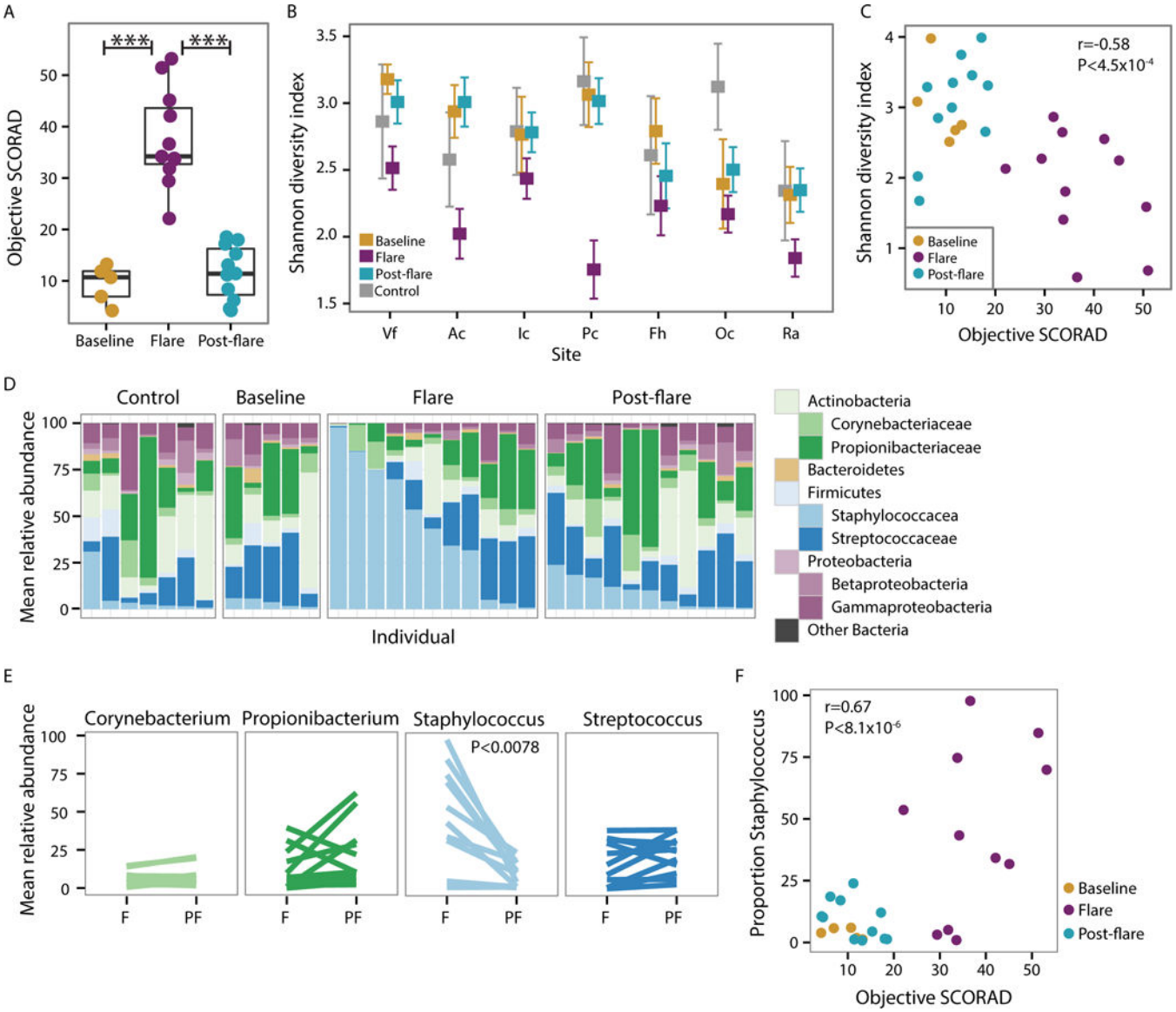

Fig. 1. Bacterial communities shift during AD disease progression

(**A**) Objective SCORAD for each patient at baseline, flare, and post-flare. Higher SCORAD corresponds to more severe disease. *** $P<0.001$ (**B**) Mean Shannon diversity +/− SEM in controls and AD disease states. Colors correspond to disease state. Volar forearm (Vf), antecubital crease (Ac), inguinal crease (Ic), popliteal crease (Pc), forehead (Fh), occiput (Oc), and retroauricular crease (Ra). (**C**) Shannon diversity versus objective SCORAD for combined antecubital (Ac) and popliteal creases (Pc) (AcPc) of AD patients. Partial correlation (adjusting for disease state). (**D**) Mean relative abundance of bacterial genera in AcPc for controls and AD disease states. (**E)** Mean relative abundance of predominant genera in AcPc for disease states, Flare (F) and Post-flare (PF). (**F**) Proportion of *Staphylococcus* versus objective SCORAD for AcPc of AD patients, partial correlation (adjusting for disease state).

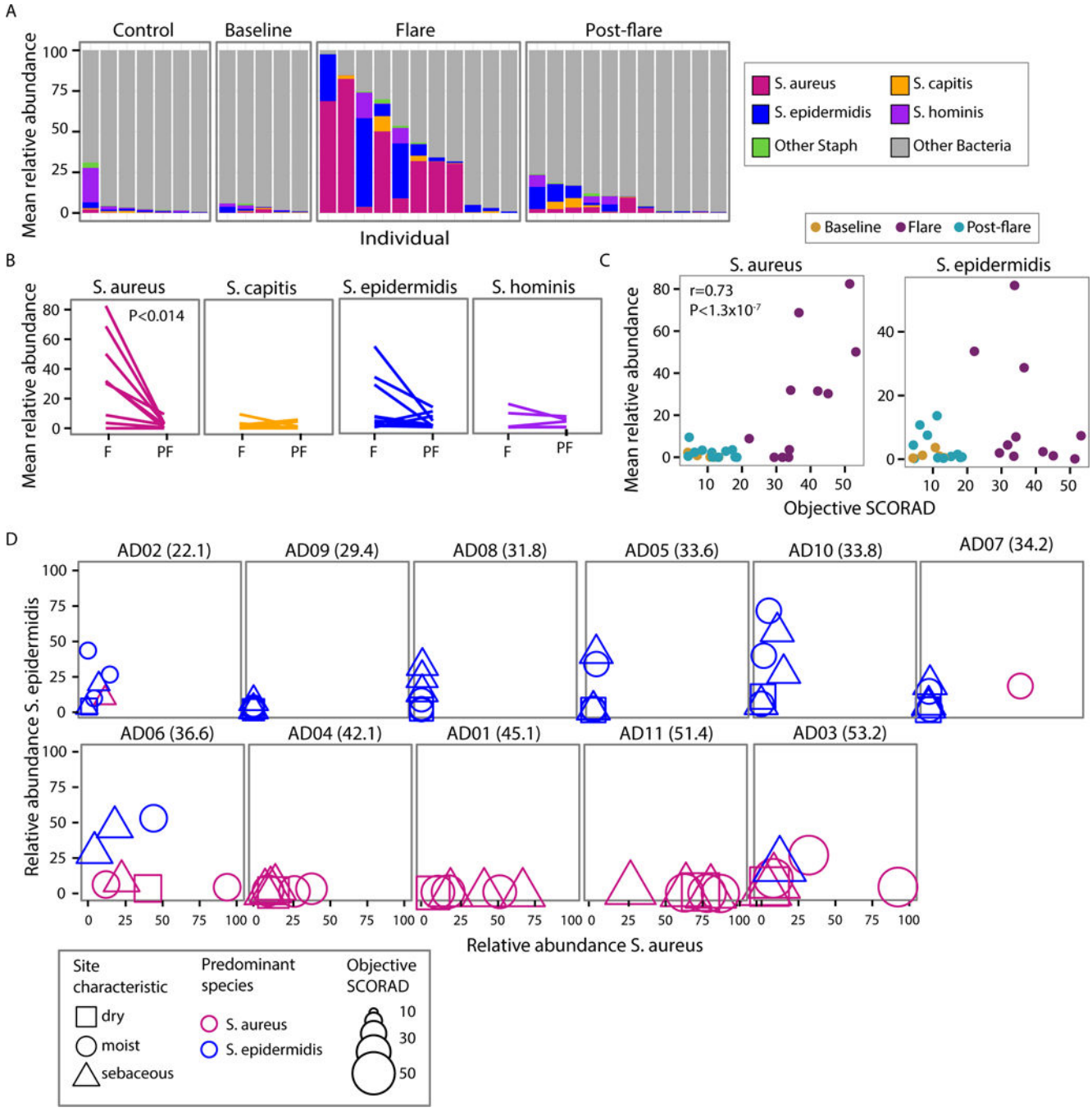

Fig. 2. Staphylococcal species increase during AD disease flare

(**A**) Mean relative abundance of staphylococcal species within the total bacterial population in combined antecubital (Ac) and popliteal creases (Pc) (AcPc) of AD patients and controls. (**B**) Mean relative abundance of most abundant Staphylococcus species in AcPc for disease states, Flare (F) and Post-flare (Pf). (**C**) Correlation of *S. aureus* (left) and *S. epidermidis* (right) mean relative abundance and objective SCORAD for AcPc of patients, partial correlation (adjusting for disease state). (**D**) Comparison of *S. aureus* to *S. epidermidis* relative abundance by patient for all sites. Patient's objective SCORAD indicated in

parenthesis. Shape corresponds to physiological characteristic of the body site, color to the predominant species, and size to the magnitude of disease severity (objective SCORAD). Patients in the top row have a higher predominance of *S. epidermidis*, while bottom row patients are *S. aureus*-predominant.

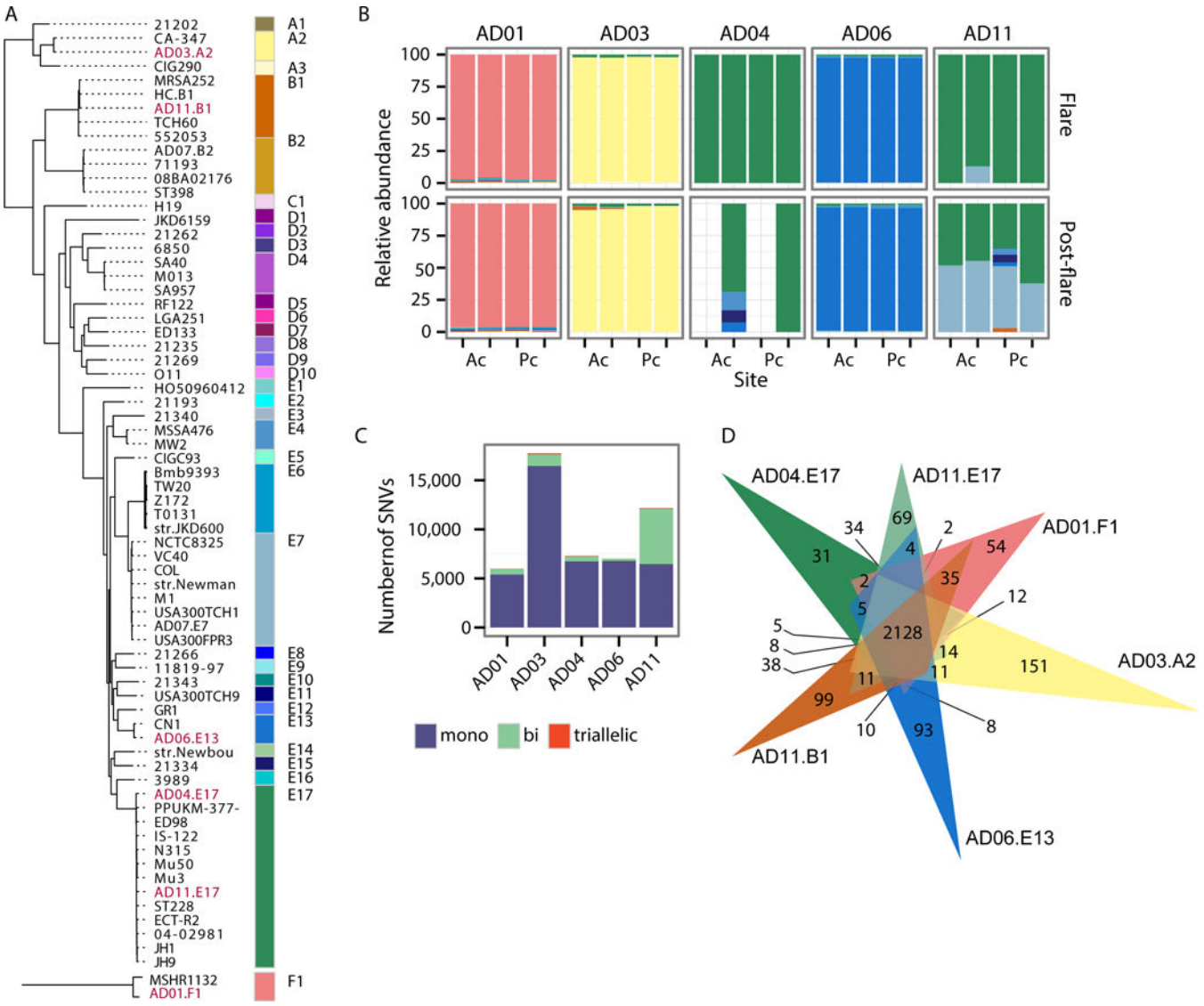

Fig. 3. *S. aureus*-predominant individuals are often colonized with a single *S. aureus* strain

(**A**) Dendogram of 61 representative *S. aureus* strains based on SNVs in the core genome. Strains labeled in red were isolated from patients in (B). Colored blocks correspond to genomes of the same clade. Phylogenetically distant clade F1 is shown as an outgroup as it was recently reclassified as *S. argenteus* (32). (**B**) For *S. aureus*-predominant individuals, *S. aureus* clade relative abundances in bilateral antecubital (Ac) and popliteal creases (Pc) for AD disease states, flare and post-flare. Colors correspond to those in (A). (**C**) For combined samples of all sites/timepoints of individuals in (B), barcharts show the number of SNVs per individual that are mono, bi, and triallelic. (**D**) Venn diagram showing the number of genes shared between isolates from patients in (B), indicated in red in (A).

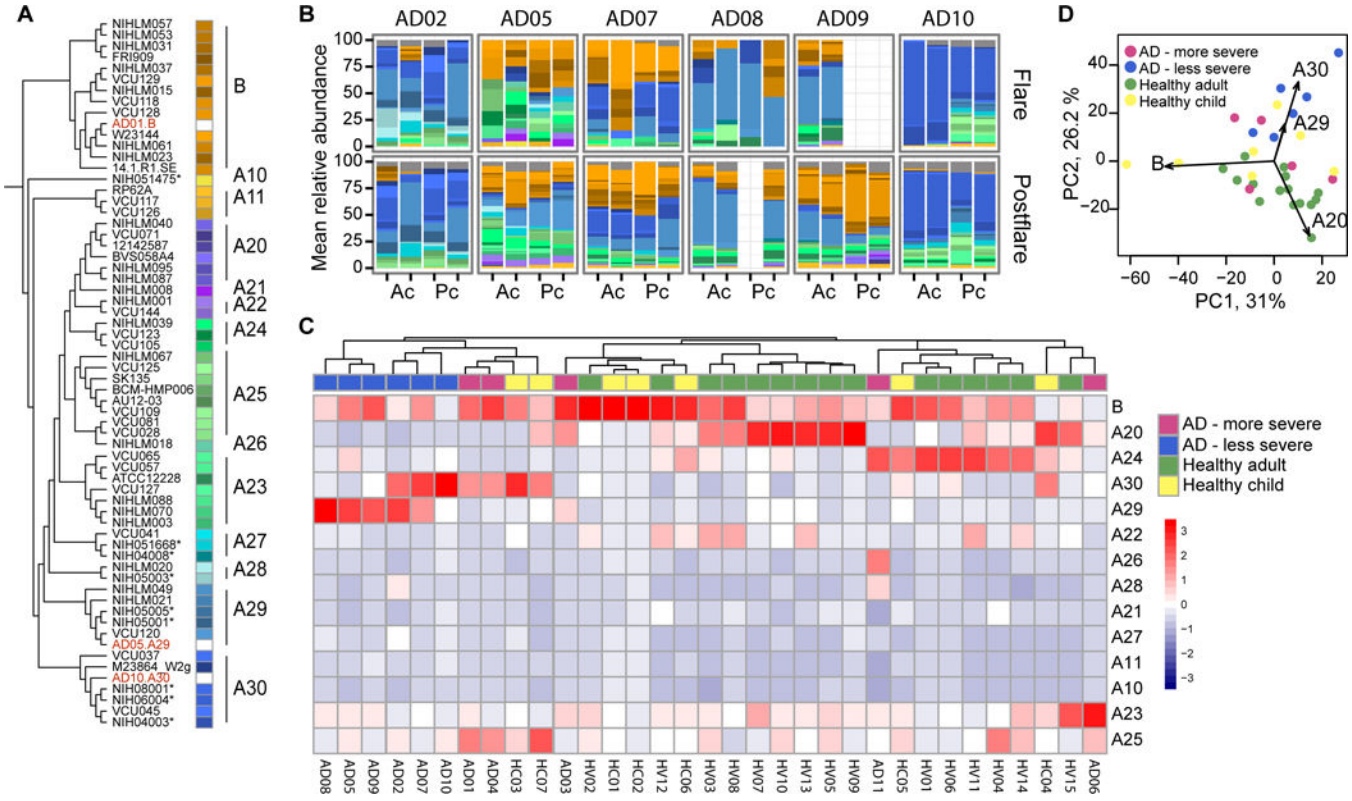

Fig. 4. *S. epidermidis*-predominant individuals are colonized by a heterogenous community of *S. epidermidis* strains

(**A**) Dendogram of *S. epidermidis* strains based on SNVs in the core genome. Strains isolated from patients in our study are labeled in red. Similar colors represent closely related strains that were grouped into 14 clades. Starred (*) isolates are nosocomial in origin (**B**) For *S. epidermidis*-predominant individuals, *S. epidermidis* strain relative abundances in combined antecubital (Ac) and popliteal creases (Pc) for AD disease states, flare and post-flare. Colors correspond to those in (A). (**C**) Heatmap shows mean relative abundance of each clade across all sites in *S. aureus* and *S. epidermidis*-predominant AD patients, healthy adults (HA), and healthy children (HC). (**D**) In principal component analysis, clades A20, A29, and A30 drive separation between *S. epidermidis*-predominant AD patients and healthy adults.

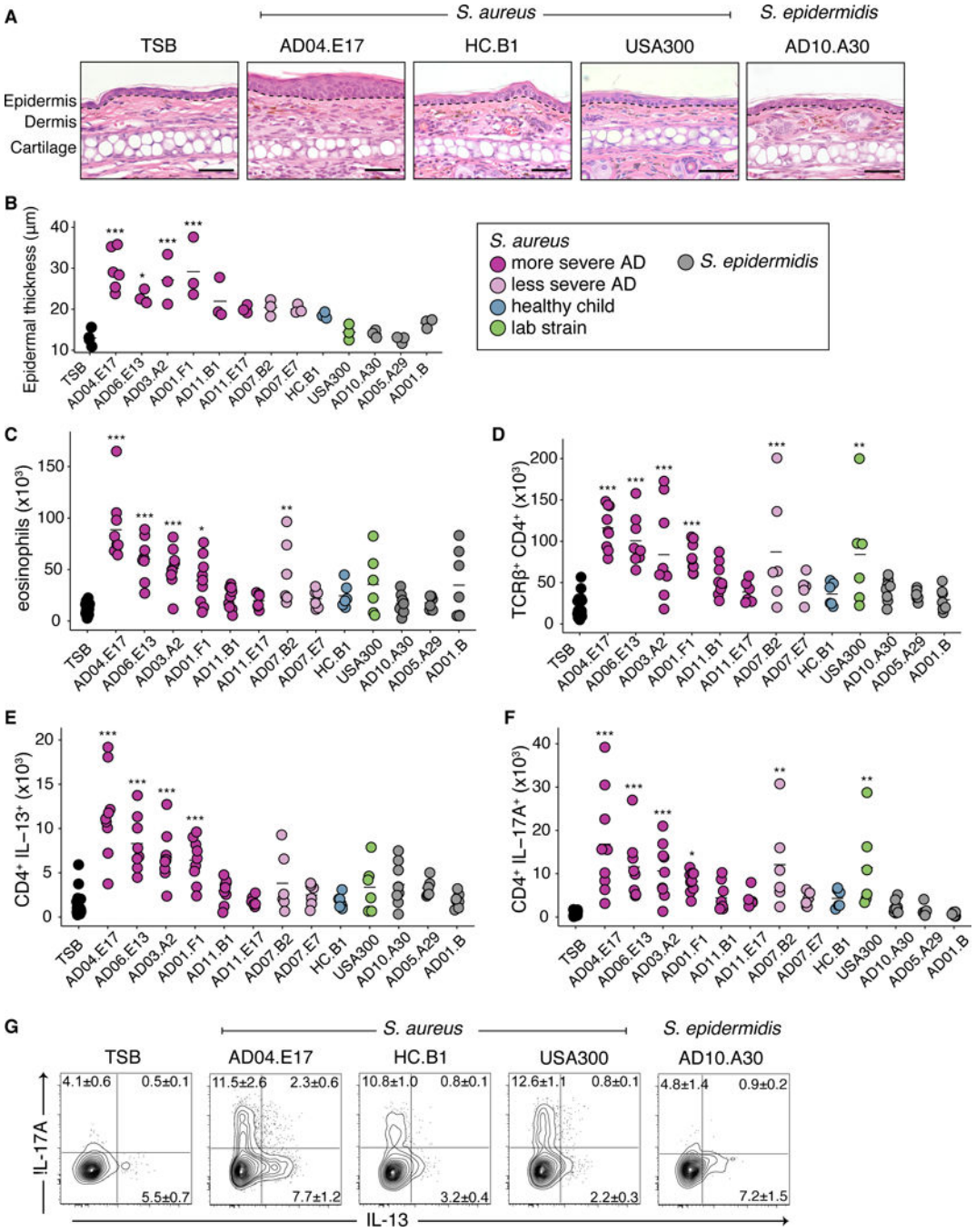

Fig. 5. Topical application of AD isolates induce AD-like immune responses in murine models

(**A**) Mice were topically associated with staphylococcal monocultures every other day 4 times before sacrifice on the 8th day. (**B**) Representative histological images of the ear pinnae of mice associated with tryptic soy broth TSB, *S. aureus* AD04.E17, HC.B1, USA300, or *S. epidermidis* A10.A30. Dotted line indicates separation between the epidermidis and dermis. Scale bar 50 μm. (**C**) Epidermal thickness of ears post topical association of patient AD isolates. Color indicates origin and species of the isolate. (**D**) Absolute numbers of skin eosinophil (**E**) Absolute numbers of skin TCRβ$^+$ CD4$^+$ cells. (**F**)

Absolute numbers of skin IL-13$^+$ CD4$^+$ cells. (**G**) Absolute numbers of skin IL-17A$^+$ CD4$^+$ cells. (**H**) Frequencies of IL-13$^+$ and IL-17A$^+$ CD4$^+$ cells from mice in (B). Results are cumulative data from 2 or 3 independent experiments, 3 mice per group. *p<0.05, **p<0.01, ***p<0.001 as calculated by ANOVA with multiple comparison correction.