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Dysbiosis and *Staphylococcus aureus* colonization drives inflammation in atopic dermatitis

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Summary

Staphylococcus aureus skin colonization is universal in atopic dermatitis and common in cancer patients treated with epidermal growth factor receptor inhibitors. However, the causal relationship of dysbiosis and eczema has yet to be clarified. Herein, we demonstrate that *Adam17*$^{fl/fl}$*Sox9*-^{Cre} mice, generated to model *ADAM17*-deficiency in human, developed eczematous dermatitis with naturally occurring dysbiosis, similar to that observed in atopic dermatitis. *Corynebacterium mastitidis*, *S. aureus*, and *Corynebacterium bovis* sequentially emerged during the onset of eczematous dermatitis, and antibiotic specific for these bacterial species almost completely reversed dysbiosis and eliminated skin inflammation. Whereas *S. aureus* prominently drove eczema formation, *C. bovis* induced robust T helper 2 cell responses. Langerhans cells were required for eliciting immune responses against *S. aureus* inoculation. These results characterize differential contributions of dysbiotic flora during eczema formation, and highlight the

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Author Contributions

T.K. and K.N. designed the study, performed experiments and wrote the paper. M.G. and H.H.K performed experiments and discussed the data. K.H., H.K. provided mice and discussed the data. M.A. discussed data and provided administrative support. H.A. and D.H.K provided mice.

microbiota-host immunity axis as a possible target for future therapeutics in eczematous dermatitis.

Introduction

Microbiome analyses in recent years have revealed that mammalian body surfaces are colonized by vast numbers of bacterial communities (Grice et al., 2009), providing impetus to explore the roles of the microbiota in normal and diseased skin. *Staphylococcus aureus* has long been known to heavily colonize skin of patients with atopic dermatitis (AD) (Leyden et al., 1974), a chronic inflammatory skin disease that develops in individuals with barrier-disrupted skin, and that leads to the progressive development of asthma and food allergy referred to as the atopic march (Spergel and Paller, 2003). Microbiome analysis has revealed temporal dybiosis dominated by *S. aureus* during AD flares (Kong et al., 2012), suggesting an important role of *S. aureus* and/or dysbiosis during active atopic inflammation.

A major cause for stratum corneum barrier disruption in AD is attributed to loss-of-function mutations in *FLG* that encodes filaggrin, a structural protein in keratinocytes/corneocytes, thereby allowing outside-in penetration of foreign antigens and subsequent sensitization (Irvine et al., 2011). Other rare genetic diseases also manifest eczematous dermatitis that are clinically nearly identical to AD, and that could represent prototypic eczema with defects in relatively well-characterized genes (Kubo et al., 2012). Among such diseases are Netherton syndrome and hyper IgE syndrome, resulting from mutations in *SPINK5* and *STAT3*, respectively (Chavanas et al., 2000; Minegishi et al., 2007). *S. aureus* is commonly isolated from skin in both of these diseases, and microbiome analysis has revealed dysbiosis with overrepresentation of *S. aureus* and *Corynebacterium* species in inflammatory skin lesions of hyper IgE syndrome (Oh et al., 2013).

Further supporting the possible involvement of dysbiosis in eczema pathophysiology, dysbiosis is implicated in other inflammatory skin conditions. Cancer patients treated with epidermal growth factor receptor (EGFR) inhibitors commonly develop eczematous rashes and pustules (Lacouture, 2006), from which *S. aureus* can be isolated (Eilers et al., 2010; Lichtenberger et al., 2013). Despite its apparent association with eczematous inflammation in AD, genetic diseases and EGFR inhibitor usage, the cause-or-effect relationship of dysbiosis and AD or eczematous dermatitis has not been determined to date. To address this issue would require a mouse model in which the microbiota alterations recapitulate human conditions.

A disintegrin and metalloproteinase 17 (ADAM17) is a transmembrane protease that cleaves a variety of membrane-bound proteins to release soluble forms (Blobel, 2005). ADAM17 plays a major role in TNFα and EGFR signaling pathways, in which pro-TNFα and pro-EGFR ligands are shed by ADAM17 from cell surfaces into active forms (Blobel, 2005). Genomic ablation of *Adam17* in mice leads to perinatal lethality (Peschon et al., 1998). ADAM17-deficiency has been recently identified in humans (Blaydon et al., 2011), defining a new genetic disorder. Importantly, described patients exhibited eczematous dermatitis and pustular lesions, and were prone to *S. aureus* infections.

Herein, we show that mice lacking *Adam17* in *Sox9*-expressing tissues including epidermis (*Adam17*$^{fl/fl}$*Sox9*-^{Cre} mice) manifested phenotypes similar to their human counterparts, exhibiting eczematous skin with disrupted barriers, elevated serum IgE and mixed T helper cell responses. Microbiome analysis of eczematous lesions in these mice revealed prominent dysbiosis that recapitulated that in human AD and eczematous dermatitis. We also demonstrate that eczematous inflammation in *Adam17*$^{fl/fl}$*Sox9*-^{Cre} mice was driven by dysbiosis, in which *S. aureus* and *C. bovis* to contribute differentially;, that dysbiosis could be induced by targeted ablation of EGFR signaling;, and that epidermal Langerhans cells mediate early immune responses to *S. aureus*.

Results

Adam17$^{fl/fl}$*Sox9*-^{Cre} mice exhibit eczematous inflammation

We generated mice that lack *Adam17* in *Sox9*-expressing tissues including epidermis (*Adam17*$^{fl/fl}$*Sox9*-^{Cre} mice; henceforth referred to as *Adam17*$^{\Delta Sox9}$ mice) initially to study ADAM17 function in bones (Horiuchi et al., 2009) and hair follicles (Nagao et al., 2012). While Sox9 is expressed in a variety of tissues including lung, pancreas, brain, gut, and kidneys, its expression in skin is mostly limited to the epidermal component (Horiuchi et al., 2009).

Consistent with previous reports that utilized mice expressing Cre recombinase driven by the keratin 14 promoter to ablate *Adam17* (Franzke et al., 2012; Murthy et al., 2012), *Adam17*$^{\Delta Sox9}$ mice exhibited dry skin around 3 weeks after birth, which progressed to eczematous lesions with intense pruritus (Figure S1A, Supplemental Movie 1), recapitulating symptoms reported in patients with *ADAM17* mutation (Blaydon et al., 2011). Lesional skin biopsies revealed mononuclear cell infiltrates including lymphocytes and mast cells (Figures 1A and S1B). Increased transepidermal water loss (TEWL) indicated barrier dysfunction (Figure 1B). Serum IgE and CCL17, the latter a T helper 2 (Th2) cell chemokine that reflects disease activity in human AD (Kakinuma et al., 2001), were elevated (Figures 1C and 1D).

Barrier disruption, eczema, and the mixture of humoral and cellular inflammation observed in *Adam17*$^{\Delta Sox9}$ mice closely resembled features of human AD and genetic diseases that mimic AD. Owing to their unimpaired survival rate, these mice were advantageous over previously reported mice with *Adam17* ablated in *Keratin 14*-expressing tissues (Franzke et al., 2012).

Consistent with the eczematous phenotype, cytokine expression analysis via flow cytometry in skin draining lymph nodes revealed increases in the numbers of Th1 and Th2 cells and prominent increases in Th17 cells (Figure 1E). Epidermal T cell composition was strikingly altered. Dendritic epidermal T cells, a resident V$\gamma5^{+}$ $\gamma\delta$ T cell subset in epidermis, were replaced by the massive infiltration of V$\gamma5^{neg}$ $\gamma\delta$ TCR^{mid} T cells and CD4^{+} T cells (Figure 1F). Approximately 20% of V$\gamma5^{neg}$ $\gamma\delta$ TCR^{mid} T cells expressed V$\gamma4$, but the majority did not (not shown). Cytokine analysis of infiltrating T cells revealed that epidermal CD4 T cells consisted of Th17 and Th22 cells, and the majority of V$\gamma5^{neg}$ $\gamma\delta$ TCR^{mid} T cells

produced interleukin-17 (IL-17) (γδT17), a fraction of which also produced IL-22 (Figure 1G).

In addition to Th1 and Th2 responses in AD, emerging reports demonstrate the contributions of Th17 and Th22 (Gittler et al., 2012; Koga et al., 2008; Nograles et al., 2009; Suarez-Farinas et al., 2013), suggesting that immune responses in AD are complex. Relative contributions of T helper subpopulations have not been explored in depth, but the prominent IL-17 responses in *Adam17*$^{\Delta Sox9}$ mice seemed atypical in the context of eczematous dermatitis. To investigate the role of IL-17 and its associated cytokine IL-23, both of which are important in psoriatic inflammation, we crossed *Adam17*$^{\Delta Sox9}$ mice to IL-17- and IL-23-deficient backgrounds. The lack of each cytokine had no effect on the onset of eczematous dermatitis (Figure S1C), demonstrating that the phenomenon observed herein was distinct from psoriasis and that IL-17 responses in *Adam17*$^{\Delta Sox9}$ mice are likely to be secondary responses. Consistently, *Adam17* $^{\Delta Sox9}$ mice exhibited striking pruritus, dry skin and eczematous dermatitis, elevated IgE concentrations and a mixture of helper T cell responses, phenotypes that are remarkably similar to symptoms generally observed in AD and eczematous dermatitis in human genetic diseases.

Adam17$^{\Delta Sox9}$ mice exhibit altered skin microbiota

Given the resemblance of the *Adam17*$^{\Delta Sox9}$ mice phenotype to human eczematous dermatitis, we swabbed and cultured skin surfaces of 8 week-old *Adam17*$^{\Delta Sox9}$ mice and WT littermates. Striking outgrowth of *S. aureus* was obtained from *Adam17*$^{\Delta Sox9}$ mice cultures, but not from WT littermates (Figure 2A). Quantifying colony-forming units showed that *S. aureus* colonization began 3 to 4 weeks after birth and increased thereafter (Figure 2B). Scanning electron microscopy and immunofluorescence microscopy revealed *S. aureus* within stratum corneum and within follicular openings (Figures 2C and Figure S2A–S2C).

Recent advances in microbiome analyses have revealed that varieties of bacterial communities inhabit mammalian body surfaces (Grice et al., 2009) and shape gut and skin immunity (Ivanov et al., 2009; Naik et al., 2012). To characterize microbiota during the onset of eczematous inflammation, we performed microbiome analyses in WT or *Adam17*$^{\Delta Sox9}$ mice over a period of 2 to 14 weeks after birth. We carried out 454 pyrosequencing of the V1–V3 region of bacterial 16S rRNA genes. All WT littermate mice exhibited identical microbiota, and underwent similar changes that began around week 8 after birth (Figure 2D), perhaps simulating bacterial community shifts observed in human physiologic development across Tanner stages (Oh et al., 2012). *S. aureus* was extremely rare among the WT skin microbiota.

Microbiota of *Adam17*$^{\Delta Sox9}$ mice were indistinguishable from WT mice at week 2 after birth, but underwent dramatic changes at week 3 to 4 (Figure 2D). Emergence of *Corynebacterium mastitidis* was initially observed, followed by *S. aureus* at approximately week 6, which appeared to coincide with the aggravation of skin symptoms (Figure S2D). Of note, *C. mastitidis* disappeared and was completely replaced by *Corynebacterium bovis*, immediately after the emergence of *S. aureus* (Figure 2D). Therefore, the skin microbiota of *Adam17*$^{\Delta Sox9}$ mice undergo a sequential change where dysbiosis starts with the emergence

of *C. mastitidis* and with *S. aureus* and *C. bovis* predominating later. The finding that dysbiosis includes *S. aureus* and *Corynebacterium* species recapitulates that which is seen in both human eczematous dermatitis and AD.

Dysbiosis drives eczematous inflammation

The above findings in *Adam17*$^{\Delta Sox9}$ mice provided a unique opportunity to address the role of dysbiosis during eczematous inflammation. Based on antibiotic sensitivity profiles of *S. aureus* (strain SAAS9) and *C. bovis* (strain CBAS9) isolates from *Adam17*$^{\Delta Sox9}$ mice, we elected to treat *Adam17*$^{\Delta Sox9}$ mice with cefazolin and enrofloxacin because of their anti-bacterial activity against the isolated strains (Table S1), established use in human (Schneider et al., 2013) or veterinarian clinical practice (Paradis et al., 1990), and solubility, which enabled continuous feeding of mice with these antibiotics through drinking water. After weaning, each group of *Adam17*$^{\Delta Sox9}$ mice was separated into cages that were given drinking water with or without antibiotics.

In striking contrast to untreated *Adam17*$^{\Delta Sox9}$ mice, antibiotic-treated mice were almost completely protected from development of eczematous lesions (Figure S3A). Clinical scores, TEWL and serum IgE concentrations also remained lower than their untreated controls (Figures 3A–3C). FACS analysis revealed a reduction in the numbers of Th2 and Th17 cells in skin draining lymph nodes (Figure 3D). Consistent with this data, serum concentrations of IL-1β, IL-6, IL-13, IL-17A and IL-17F were decreased after antibiotic treatment, although CCL17/TARC remained high (Figure S3B). Antibiotics also normalized epidermal γδ T cell constituents (Figure S3C). $CD4^+$ T cell numbers in epidermis of antibiotic-treated *Adam17*$^{\Delta Sox9}$ mice remained high, but these remaining $CD4^+$ T cells produced less IL-4 and did not produce IL-17A or IL-22 (Figure 3E and Figure S3D). A potential explanation is that the $CD4^+$ T cells were attracted to epidermis via CCL17 that remained highly expressed, but were not primed to produce cytokines owing to the decrease in epidermal bacterial load.

Microbiome analysis revealed higher bacterial diversity in antibiotic-treated *Adam17*$^{\Delta Sox9}$ mice than in untreated mice, and dramatic reductions in the proportions of *S. aureus* were observed (Figures 3F and G and Figure S3E). *C. bovis* proportions were also reduced, but to a lesser extent when compared to the changes observed for *S. aureus* (Figures 3F and 3G and Figure S3E). The bacterial community structure in untreated *Adam17*$^{\Delta Sox9}$ mice and controls diverged over time but was more similar after antibiotic treatment (Figures 3G and 3H).

We further investigated the association of dysbiosis with eczematous inflammation by performing protocol crossover studies. A group of *Adam17*$^{\Delta Sox9}$ mice was left untreated up to week 10 after birth, and then subsequently received antibiotic therapy. Conversely, another group of *Adam17*$^{\Delta Sox9}$ mice was maintained on antibiotics from week 3 to 10, which were discontinued thereafter (Figure S4A). Strikingly, initiation of antibiotics in previously untreated *Adam17*$^{\Delta Sox9}$ mice extinguished pre-established eczematous dermatitis and inflammation (Figures 4A–C and Figure S4A) and reversed dysbiosis (Figure 4D and Figure S4B), indicating that targeting dysbiosis had a therapeutic effect. Furthermore, although mice receiving continuous antibiotics were prevented from developing eczematous lesions,

withdrawal of antibiotics led to marked skin inflammation with rapid emergence of *C. bovis* and *S. aureus* (Figures 4A–D and Figures S4A and S4B). These data collectively establish dysbiosis as a pivotal event during the onset of eczematous dermatitis in *Adam17*$^{\Delta Sox9}$ mice.

S. aureus and *Corynebacterium* species contribute to eczematous dermatitis formation

We next explored which of the three bacterial species that constituted dysbiotic flora in *Adam17*$^{\Delta Sox9}$ mice contributed to the pathogenesis of eczematous inflammation. Meta-analysis of clinical score, TEWL, and IgE in mice described in Figures 1 and 3 was performed. As anticipated from the association of *S. aureus* in AD, and concordant with our observation that *S. aureus* emerged immediately prior to the onset of eczematous dermatitis (Figure S2D), formation of eczematous dermatitis in *Adam17*$^{\Delta Sox9}$ mice correlated with the proportion of *S. aureus* and *C. bovis* (Figures S5A and S5B). IgE concentrations correlated with only *C. bovis* frequency (Figure S5A). No positive correlation of *C. mastitidis* with any of the factors studied herein was found.

To assess the contribution of each bacterial species *in vivo*, we utilized *Adam17*$^{\Delta Sox9}$ mice that were pre-treated with antibiotics. *Adam17*$^{\Delta Sox9}$ mice treated with the antibiotic cocktail showed minimal signs of eczema (Figures S3A and S4A). After termination of treatment, there exists an almost 2-week period before overt appearance of eczematous dermatitis. Taking advantage of this window, we inoculated SAAS9, CBAS9, or a *C. mastitidis* strain (CMAS9, isolated from *Adam17*$^{\Delta Sox9}$ mice) onto *Adam17*$^{\Delta Sox9}$ mice immediately after termination of antibiotics (Figure S5C). Strikingly, and consistent with the meta-analysis, inoculation of mice with SAAS9 induced the most severe eczematous dermatitis (Figures 5A and S5D). Inoculation of CBAS9 also induced enhanced eczematous dermatitis, albeit to a lesser extent than SAAS9 (Figures 5A and S5D). Clinical scores of mice that were inoculated with CMAS9 were variable and did not reach statistical significance when compared to un-inoculated control *Adam17*$^{\Delta Sox9}$ mice (Figures 5A and S5D).

T helper responses in skin-draining lymph nodes were analyzed. Surprisingly, SAAS9-inoculation did not have an impact on the numbers of Th1 or Th2 cells (Figure 5B). In contrast, increased Th1 and Th2 responses, in particular the latter, were observed in skin-draining lymph nodes of mice inoculated with CBAS9 (Figure 5B). Although both SAAS9 and CBAS9 showed trends for enhancing Th17 responses, they did not reach not statistical significance. CMAS9 had no effect on T helper responses that were analyzed.

These results identified *S. aureus* as potent inducer of eczematous dermatitis via an immunological pathway that has yet to be determined. *C. bovis* prominently enhanced Th2 responses, providing explanation for elevated serum IgE concentrations in *Adam17*$^{\Delta Sox9}$ mice. Taken together, *S. aureus* and *C. bovis* contribute to different aspects of eczematous inflammation in this model.

Ablation of EGFR-signaling allows dysbiosis

Important roles of EGFR signaling in regulating skin immune homeostasis have begun to be characterized, either in the context of AD (Saaf et al., 2012; Zhang et al., 2014) or EGFR-inhibitors (Lacouture, 2006). EGFR ligands are major substrates of ADAM17. ADAM17

cleaves the membrane-bound pro-forms of EGFR ligands including heparin-binding EGF-like growth factor and TGFα, and releases soluble forms to activate EGFR signaling pathways. It was recently shown that conditional ablation of EGFR from epidermis led to a skin inflammatory phenotype that was associated with barrier defects (Lichtenberger et al., 2013; Mascia et al., 2013). We therefore explored whether impaired EGFR signaling downstream of ADAM17 contributed to dysbiosis and eczematous dermatitis formation.

We generated *Egfr*$^{fl/fl}$*Sox9*-^{Cre} (*Egfr*$^{\Delta Sox9}$) mice that did not express *Egfr* in Sox9-expressing tissues. Consistent with other reports, *Egfr*$^{\Delta Sox9}$ mice exhibited eczematous phenotypes that were similar to, but slightly less severe than, those in *Adam17*$^{\Delta Sox9}$ mice (Figures 6A and S6). TEWL and serum IgE concentrations were elevated in comparison to WT mice (Figures 6B and 6C). Inflammatory profiles were also similar to *Adam17*$^{\Delta Sox9}$ mice, where *Egfr*$^{\Delta Sox9}$ mice exhibited mixed Th1, 2 and 17 accumulations in lymph nodes and Th17, Th22 and Tγδ17 inflammation in skin (Figures 6D and 6E) The numbers of IL-22-producing epidermal T cells were not as prominent in *Egfr*$^{\Delta Sox9}$ mice as in *Adam17*$^{\Delta Sox9}$ mice (Figure 6E).

Skin swab cultures from 8-week-old *Egfr*$^{\Delta Sox9}$ mice isolated *S. aureus* (Figure 6F and 6G), and microbiome analysis revealed the emergence of *Corynebacterium* spp and *S. aureus* (Figure 6H). Dysbiotic changes were less prominent than those of *Adam17*$^{\Delta Sox9}$ mice, but nevertheless document impairment of EGFR signaling as an important pathway downstream of ADAM17 that allows the overgrowth of *S. aureus* and *Corynebacterium* species.

Taken together, ADAM17 regulates homeostasis of cutaneous microbial communities in an EGFR-dependent manner. Further elucidation of mechanisms downstream of EGFR signaling should provide information on molecules and cells that could be considered to be potential targets to normalize dysbiosis.

Langerhans cells mediate innate responses to *S. aureus*

In contrast to a colitis model in which gut dysbiosis and disease can be transferred to co-housed WT mice (Elinav et al., 2011), co-housing WT mice with *Adam17*$^{\Delta Sox9}$ mice only led to temporal colonization of the former with *S. aureus* and did not transfer skin disease (data not shown). This suggested that *S. aureus* alone was insufficient to cause eczematous inflammation, and that it was incapable of persistently colonizing normal skin. Therefore, to determine whether *S. aureus* could induce immune responses through an impaired stratum corneum barrier, we utilized *Flg*−/− mice that have a defined barrier defect (Kawasaki et al., 2012). Filaggrin is a major structural protein in the stratum corneum, and *FLG* mutations are associated with human AD (Palmer et al., 2006). However, *Flg*−/− mice do not develop spontaneous dermatitis under normal SPF conditions, but exhibit enhanced percutaneous sensitization (Kawasaki et al., 2012). Epidermal tight junctions are intact in these mice (unpublished data).

S. aureus (SAAS9) isolated from *Adam17*$^{\Delta Sox9}$ mice were topically inoculated onto the shaved back skin of *Flg*$^{-/-}$ and WT mice. Although topical inoculation did not induce overt dermatitis, it induced robust skin infiltration of T_H17 and γδT17 cells into epidermis in

Flg$^{-/-}$, but not WT mice (Figure 7A), demonstrating that barrier disruption in the stratum corneum was sufficient for enhanced immune responses to topically inoculated *S. aureus*.

Langerhans cells (LC) are unique dendritic cells in the epidermis (Merad et al., 2008). Through a series of studies, we have demonstrated that LC take up microbial antigens that have breached the stratum corneum, but exist outside of tight junctions, by extending their dendrites through intact tight junction barrier and induce antigen-specific humoral (IgG_1) responses (Nagao et al., 2009; Kubo et al., 2009; Ouchi et al., 2011). When the epidermal barriers are breached during Candida infection, LC are capable of inducing Th17 responses (Igyarto et al., 2011).

During dysbiosis that occur in stratum corneum (Figures S2A–C), LC are advantageously positioned to acquire those bacteria through epidermal tight junctions. To determine if LC contributed to immunity against *S. aureus*, we generated *Flg*$^{-/-}$ mice that lacked LC, by crossing them to mice that express diphtheria toxin fragment A driven by the human langerin promoter (Langerin-DTA mice) (Kaplan et al., 2005). Of note, Th17 and γδT17 responses induced by topical SAAS9-inoculation were abrogated in *Flg*$^{-/-}$ Langerin-DTA mice (Figure 7A), demonstrating a role for LC in mediating IL-17 responses against *S. aureus*.

Because topical SAAS9 exposure did not generate dermatitis, we inoculated SAAS9 via an occlusive dressing technique that led to the formation of visible dermatitis in WT mice (Figure S7) that lasted for approximately 3 days. Consistent with the immune responses in the topical exposure protocol, dermatitis was aggravated in *Flg*$^{-/-}$ mice. However, dermatitis was attenuated in *Flg*$^{-/-}$ Langerin-DTA mice, and IL-17A responses were nearly abrogated (Figures 7B,7C and S7). IL-22 responses were not observed in any setting (Data not shown). The transient nature of dermatitis in this assay indicates that *S. aureus* and barrier disruption are not sufficient to cause chronic disease. Nevertheless, these data demonstrated that LC facilitated innate immune responses against *S. aureus*, presumably by acquiring components of *S. aureus* through the tight junction barrier. This process likely represents an early immunological event in the initial phases of dysbiosis and skin inflammation.

Discussion

Eczematous inflammation in *Adam17*$^{\Delta Sox9}$ mice occurred in conjunction with a striking shift in microbiota, in which *S. aureus* and *Corybacterium* spp were major constituents. This dysbiotic pattern closely recapitulated those seen in human AD (Kong et al., 2012) and primary immunodeficiencies with eczematous dermatitis (Oh et al., 2013). In particular, *S. aureus* colonization is universal in AD patients, and it has been long debated whether emergence of *S. aureus* is a secondary by-product of chronic inflammation or whether it is a primary component of pathogenesis that actively contributes to eczema development. In *Adam17*$^{\Delta Sox9}$ mice, dysbiosis was a pivotal event during eczematous dermatitis formation, and antibiotics-mediated normalization of dysbiosis both prevented onset and suppressed active inflammation. Furthermore, bacterial inoculation experiments in *Adam17*$^{\Delta Sox9}$ mice revealed that eczematous inflammation could be accelerated by both *S. aureus* and *C. bovis*

(*S. aureus* > *C. bovis*). *C. bovis* was the only agent that induced prominent Th2 responses, suggesting differential contribution of *S. aureus* and *C. bovis* to the complex eczematous phenotype. Generation of gnotobiotic mice would be required to determine whether each agent is sufficient to induce full-blown disease in the absence of each other, and to determine immunological pathways induced by *S. aureus* inoculation that are critical for eczematous dermatitis formation. Although a role for *C. mastitidis* was not revealed in the current study, it is attractive to hypothesize that this organism might enhance the growth of *S. aureus* and *C. bovis*.

The importance of IL-17 responses is well established in psoriasis in both mice and humans (Lowes et al., 2014). Recent studies report the involvement of IL-17 responses also in human AD (Koga et al., 2008; Suarez-Farinas et al., 2013), but its relative contribution to AD pathogenesis is not yet determined. Although *Adam17*$^{\Delta Sox9}$ mice did not exhibit macroscopic features that were reminiscent of psoriasis, the robust IL-17 responses prompted us to exclude psoriatic inflammation. The lack of improvement in *Adam17*$^{\Delta Sox9}$ mice in both IL-17 and IL-23 backgrounds indicate that the pathophysiology of the skin inflammation is distinct from that of psoriasis and that these cytokines are not essential during an eczematous response. It is possible that the IL-17 responses observed here arise secondarily to dysbiosis, and perhaps, might be a consequence of utilizing mouse models.

While mutations in the gene encoding the structural stratum corneum protein Filaggrin are considered major predisposing factors in “classic” AD (Irvine et al., 2011), a variety of other genes are involved, and it is possible that further elucidation of involved genes might subdivide this heterogeneous disease. Whether or not eczematous dermatitis seen in *Adam17*$^{\Delta Sox9}$ mice can be extrapolated to human diseases remains to be determined. Importantly, the mouse model studied herein is an actual counterpart of a rare human disease that manifests eczematous dermatitis (Blaydon et al., 2011), and therefore represents a prototypic eczematous dermatitis model. Dry skin, pruritus, elevated serum IgE, and naturally occurring dysbiosis in *Adam17*$^{\Delta Sox9}$ mice recapitulate the most important aspects of human eczematous dermatitis and AD.

A systematic review of human studies failed to provide strong evidence for recommending interventions to reduce *S. aureus* in AD (Bath-Hextall et al., 2010). However, outcomes of antibiotic therapy in humans may be variable due to unconfirmed presence of dysbiosis at the start of the studies and as of yet suboptimal combinations and/or dose of antibiotics. From this viewpoint, future studies might require pre-treatment microbiome analyses. Furthermore, intensified antibiotic cocktails might be necessary to target skin-colonizing *S. aureus*, as is done for *Helicobacter pylori* eradication (Chey and Wong, 2007; Malfertheiner et al., 2007). Thus, antibiotic data currently available for humans is inconclusive and requires further evaluation.

We demonstrated that targeting dysbiosis via antibiotic cocktails had dramatic effects on eczematous lesions of *Adam17*$^{\Delta Sox9}$ mice. However, administering an antibiotic cocktail for a prolonged period of time, as we have done experimentally, is not likely to be a practical approach in patients. Long-term oral antibiotic therapy would have undesirable effects on microbiota in the gut and other distal sites (Cho et al., 2012; Willing et al., 2011) and could

also select antibiotic-resistant bacteria (Sommer and Dantas, 2011; Wright, 2007). Meanwhile, bleach baths, a common clinical practice that is receiving increasing scientific interest, is recommended for patients with moderate to severe AD to reduce disease severity (Eichenfield et al., 2014; Huang et al., 2009; Leung et al., 2013), and could potentially be targeting dysbiosis. In combination with the findings in the current work, mechanisms of this classic therapy might deserve further exploration. The finding that dysbiosis occurs in part via impaired EGFR signaling might provide foundation for novel strategies to modify the skin microbiota.

In conclusion, the findings in this study document dysbiosis as a pathological factor that can drive eczematous inflammation in mice. Utilizing this unique model, it will be possible to further explore molecular mechanisms in epidermis that result in dysbiosis and inflammation, and to design strategies that normalize them long-term. Accomplishment of these in a skin-specific manner will lead to safe and effective therapies and should have profound impact on the treatment of AD, as well as other forms of eczematous dermatitis and perhaps skin rash associated with EGFR inhibitor use.

Experimental Procedures

Details of experimental procedures on microbiome analysis are included in Supplemental Information.

Mice

Adam17$^{\Delta Sox9}$ mice and *Egfr*$^{\Delta Sox9}$ mice were generated by crossing *Adam17*$^{flox/flox}$ mice or *Egfr*$^{flox/flox}$ mice (kind gift from Dr. David Threadgill, University of North Carolina) with *Sox9*-^{Cre} mice as previously described (Horiuchi et al., 2009; Nagao et al., 2012). Mice were backcrossed into the C57BL/6 background for at least 6 generations. *IL-17*$^{-/-}$ and *IL-23p19*$^{-/-}$ mice were generously provided by Dr. A. Yoshimura (Keio University School of Medicine, Tokyo, Japan). The generation of *Flg*$^{-/-}$ mice and Langerin-DTA mice has been described (Kaplan et al., 2005; Kawasaki et al., 2012). All mice were bred and housed in a specific pathogen free condition. All animal experimental procedures and study protocols were approved by the Ethical Committee for Animal Experiments of the Keio University School of Medicine.

Preparation of epidermal cell suspensions

Epidermal cell suspensions were prepared from mouse trunk skin. Shaved trunk skin was incubated with 0.15% trypsin and 0.27 mM EDTA at 37°C for 45 minutes. Epidermal cells were scraped off in 5% FBS PBS, washed and then filtered through cell strainers (BD).

Flow cytometry analysis and antibodies

For cytokine analysis, cell suspensions were incubated for 5 hours in complete RPMI with 50 ng/ml PMA and 500 ng/ml Ionomycin in the presence of 5 μg/ml Brefeldin A. Cells were stained with LIVE/DEAD Fixable Dead Cell Stain Kits (Invitrogen) and surface markers, followed by fixation and permeabilization with BD Cytofix/Cytoperm (BD) and intracellular cytokine staining. The following antibodies from Biolegend or eBioscience were used for

analysis: anti-CD45 (30-F11), anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-TCRγδ (GL3), anti-IL-17A (TC11-18H10.1), anti-IL-4 (11B11), anti-IFN-γ (XMG1.2) and anti-IL-22 (1H8PWSR).

Antibiotic treatment

Mice were treated with 0.5 mg/ml cefazolin (Nichi-Iko Pharmaceutical Co., Ltd.) and 0.5mg/ml enrofloxacin (Bayer HealthCare) in drinking water.

Histological analysis and immunofluorescence microscopy

Skin from the facial area of sacrificed mice were taken for hematoxylin and eosin and toluidine blue staining Immunofluorescence microscopy was performed to detect *S. aureus* with biotinylated rabbit polyclonal anti-*S. aureus* antibody (abcam) which was detected with AlexaFluor 488-conjugated anti-biotin secondary antibody (Invitrogen).

Electron microscopy

For scanning electron microscopy, skin taken from the facial area was fixed in 2% glutaraldehyde, post-fixed with osmium tetroxide, replaced with isoamyl acetate and were observed by SV6600 (Hitachi). For transmission electron microscopy, skin samples were pre-fixed with 2% glutaraldehyde, post-fixed in osmium tetroxide and were embedded in epoxy resin. Ultrathin sections were prepared and were observed by JEM-1230 (JEOL).

IgE and Cytokine analysis

Serum total IgE were measured by Mouse IgE ELISA quantitative set (Bethyl Laboratories). Cytokine concentrations were measured by Milliplex Map Kit (Millipore), and serum CCL17/TARC were detected by Mouse CCL17/TARC Quantikine (R&D Systems).

Clinical score

Clinical severity of skin lesions was scored with a method adapted from a scoring index of canine atopic dermatitis (Plant et al., 2012). In brief, the clinical score was measured by assessing the severity and extent of lesions (erythema, excoriation, erosion, alopecia, lichenification and hyperpigmentation) at head and pinnae, forefeet, hind feet, ventral thorax and axillae, ventral abdomen and inguinal, and graded as 0 (none), 1 (mild), 2,3 (moderate), or 4,5 (severe and extensive lesions). The total index ranges from 0 to 50.

Trans epidermal water loss (TEWL)

TEWL was measured with a VAPOSCAN AS-VT100RS machine (Asahi Biomed).

Bacterial culture

Cultures were obtained with swabs from a cheek of mice and plated on Mannitol salt agar with egg yolk (BD) or Trypticase soy agar with 5% sheep blood (BD). To measure CFUs of *S. aureus*, a standard plate count method was performed. Briefly, skin swabs from a cheek of mice (1 cm^2) were placed into 1 ml of PBS, and serial dilutions were prepared. 100 μl of each dilution was transferred on Mannitol salt agar with egg yolk plates (BD), and colonies

were counted after 2 day-incubation. CFUs were calculated by dividing the number of colonies per plate by the dilution factor.

Bacterial isolation and inoculation onto *Adam17* ^{Sox9} mice

C. mastitidis and *C. bovis* were isolated from *Adam17* ^{Sox9} mice on Trypticase soy agar with 5% sheep blood plates (BD), and *S. aureus* was isolated by culturing on Mannitol salt agar with egg yolk plates (BD), for consistency. Isolated strains were designated as SAAS9 (*S. aureus*), CBAS9 (*C. bovis*), and CMAS9 (*C. mastitidis*). All analyzed *S. aureus* isolates had sequences that were identical to that of ST88 clone via multilocus sequence typing (MLST) (http://saureus.mlst.net), therefore likely representing a single clone. 10 colonies of each bacteria species were picked and dissolved in 0.4 ml sterile PBS. Bacteria containing PBS or control PBS were inoculated with cotton swabs on facial area of mice. The mice were pre-treated with antibiotics from 3 weeks after birth to 8–10 weeks, and inoculation of bacteria was started after withdrawal of antibiotic treatment. The inoculation was performed from day 0 to day 6, and the mice were analyzed on day 8.

SAAS9 inoculation onto *Flg*$^{-/-}$ mice

SAAS9 was placed into tryptic soy broth (BD) and grown overnight at 37°C in a shaking incubator. Exponential-phase bacteria were obtained after a one-hour subculture of a 1:50 dilution of the overnight culture. Bacterial concentrations were estimated by measuring the absorbance of at 600 nm. Bacterial cells were pelleted and washed 3 times in PBS. 4×10^7 colony forming units of SAAS9 in 100μl of PBS were topically inoculated on shaved back skin of mice. SAAS9 inoculation was performed at day 1, 3, 5, 7 and 9, and the mice were examined at day 10. For inoculation via occlusive dressing, 4×10^7 colony forming units of SAAS9 were applied, and the back skin of mice was covered with Tegaderm™ Roll (Sumitomo 3M Limited). The dressing was removed at day 4, and the mice were examined at day 7. Area of skin lesions were photographed and measured by Photoshop CS5.1 (Adobe).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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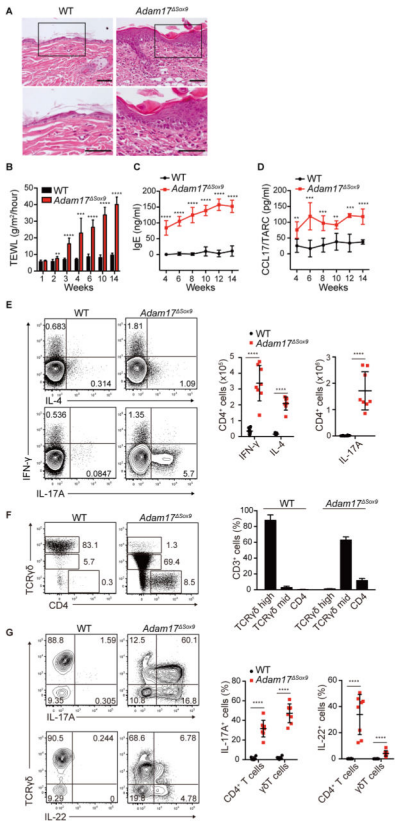

Figure 1. Eczematous inflammation in ADAM17-deficient skin

(A) Histopathology of skin biopsy from WT and *Adam17*$^{fl/fl}$*Sox9*-^{Cre} (*Adam17*$^{\Delta Sox9}$) mice. Scale bars, 50 μm. (B–D) TEWL, serum IgE and serum CCL17/TARC values in WT and *Adam17*$^{\Delta Sox9}$ mice (*N*=8). (E) Flow cytometry analysis of IFN-γ, IL-4 and IL-17A production in CD4^{+} cells from lymph nodes, and (F, G) IL-17A and IL-22 production by CD3^{+} cells in epidermis of WT and *Adam17*$^{\Delta Sox9}$ mice (*N*=8). TCRγδ^{high} cells represent dendritic epidermal T cells, and TCRγδ^{mid} cells represent γδ T cells that have infiltrated epidermis from the dermis. Data in B–G are shown as mean ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 as determined by Student's t test. See also Figures S1.

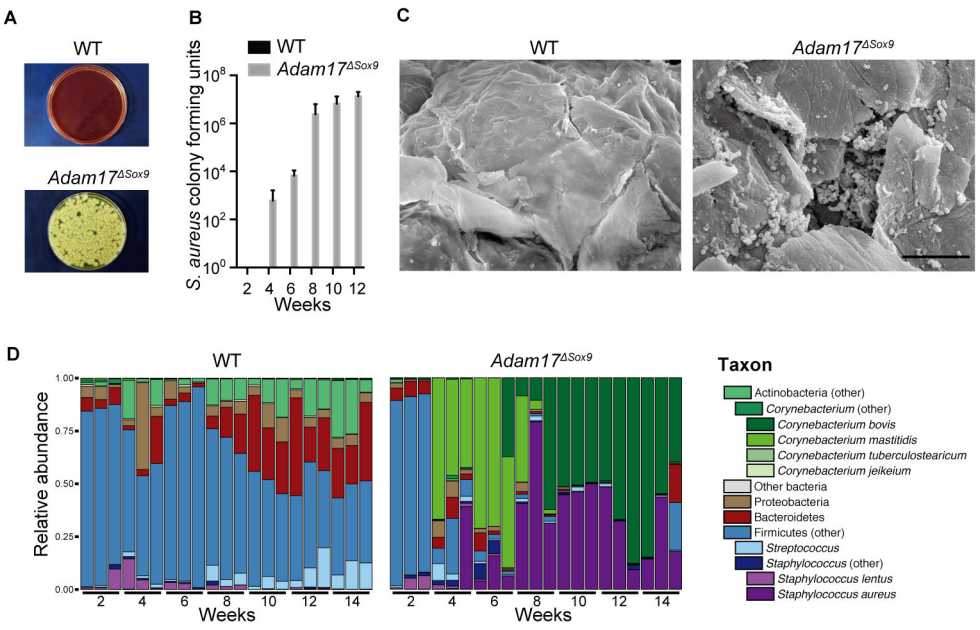

Figure 2. *Staphylococcus aureus*-associated dysbiosis in *Adam17*$^{\Delta Sox9}$ mouse skin

(A, B) Quantification of *S. aureus* cultured from facial skin swab samples of WT and *Adam17*$^{\Delta Sox9}$ mice (*N*=8, mean ± SD). (C) Scanning electron microscopy on skin surface of WT and *Adam17*$^{\Delta Sox9}$ mice. Scale bars, 10 μm. (D) Microbiota of WT and *Adam17*$^{\Delta Sox9}$ mice analyzed at indicated time points after birth. Relative abundance of order-genera that represented > 1% of total 16S rRNA gene sequences with additional speciation of *Corynebacterium* and *Staphylococcus* genus. Results represent two independent experiments (*N*=3). See also Figures S2.

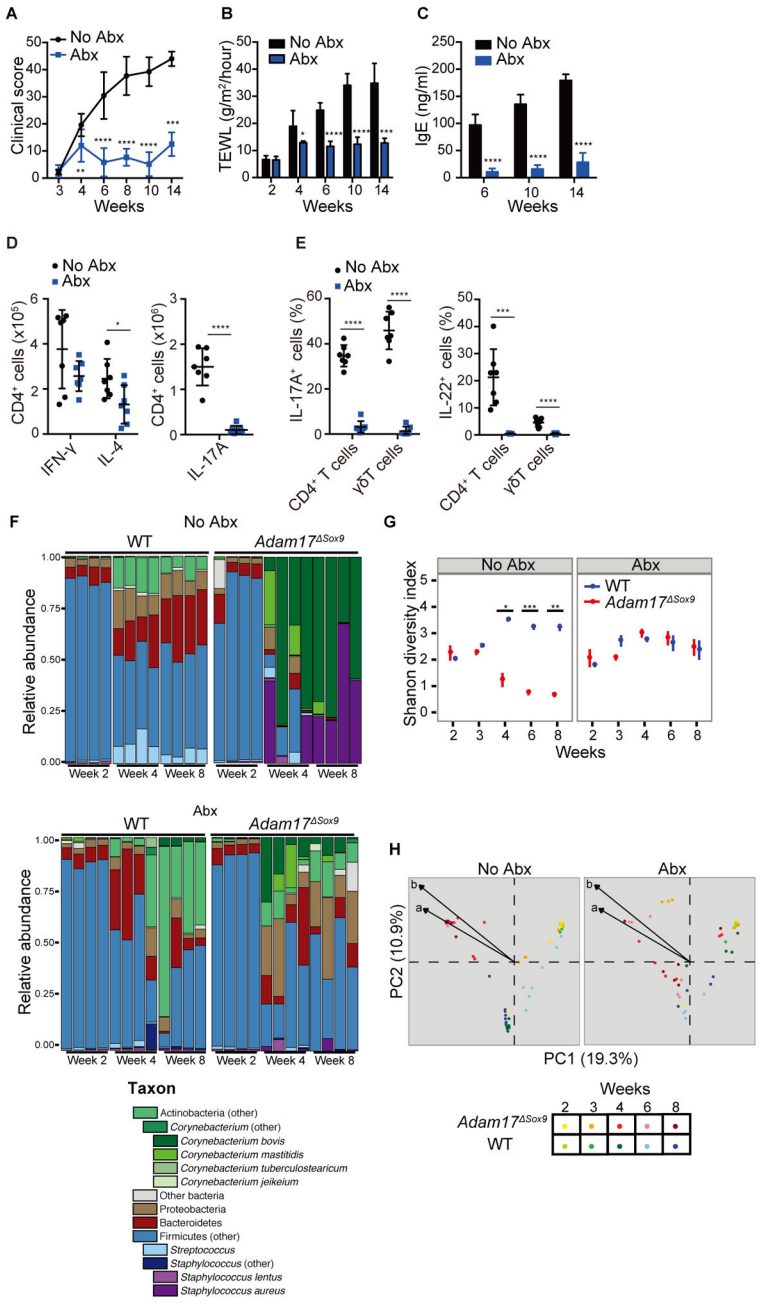

Figure 3. Antibiotics prevent eczematous inflammation and conserves bacterial diversity

Adam17$^{\Delta Sox9}$ mice were either treated or untreated with antibiotics cocktail (Abx) since weaning. See also Figure S3. (A–C) Clinical score, TEWL and serum IgE concentrations (*N*=7). A C shown as mean ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P* <0.0001 as determined by Student's t test. (D) Flow cytometry analysis of IFN-γ, IL-4 and IL-17A production in CD4$^+$ cells from lymph nodes, and (E) IL-17A and IL-22 production by CD3$^+$ cells in epidermis of WT and *Adam17*$^{\Delta Sox9}$ mice (*N*=7). (F) Microbiota of Abx-treated and untreated *Adam17*$^{\Delta Sox9}$ and WT mice at indicated time points after birth. Relative abundance plots of order-genera that represented >1% of total 16S rRNA gene sequences

with additional speciation of *Corynebacterium* and *Staphylococcus* genus of 4 representative mice for each time point. Results are representative of two experiments. (G) Shannon diversity index of Abx-treated and untreated *Adam17*$^{\Delta Sox9}$ and WT mice from week 2 to 8 after birth. Each time point represents mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 determined by Student's t test and adjusted for multiple comparison by Benjamini and Hochberg correction. (H) Principal coordinates analysis (PCoA) of the microbial community structure (Theta similarity index) of Abx-treated and untreated *Adam17*$^{\Delta Sox9}$ and WT mice. A shorter distance between points indicates higher similarity between the microbiome of samples represented by these points. Biplot arrows indicate the two most significant unique consensus taxonomies contributing to variation along axes 1 and 2 as determined by Spearman correlation. Length of biplot arrows reflects the contribution of that taxa to the top two axes: a, *S. aureus* (r=−0.73, *P<1e-15*); b, *C. bovis* (r=−0.77, *P<1e-15*). Spearman correlations and P-values refer to axis 1.

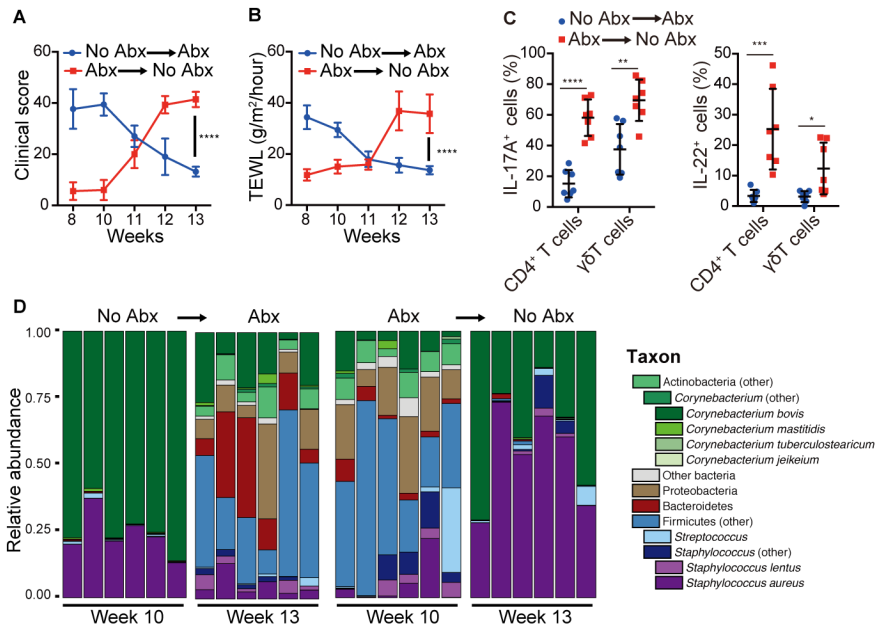

Figure 4. Antibiotics have therapeutic effect in *Adam17*$^{\Delta Sox9}$ mice

Adam17$^{\Delta Sox9}$ mice were untreated from 3~9 weeks after birth, and were put on antibiotics from 10~13 weeks, or vice versa. See also Figure S4. (A, B) Clinical score and TEWL in *Adam17*$^{\Delta Sox9}$ mice during the crossover protocol (*N*=7). (C) IL-17A and IL-22 production by CD3^{+} cells in epidermis of *Adam17*$^{\Delta Sox9}$ mice during the crossover protocol (*N*=7). (D) Relative abundance of order-genera that represented >1% of total 16S rRNA sequences with additional speciation of *Corynebacterium* and *Staphylococcus* genus before and after crossover. The data in A–C are shown as the mean ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 determined by Student's t test.

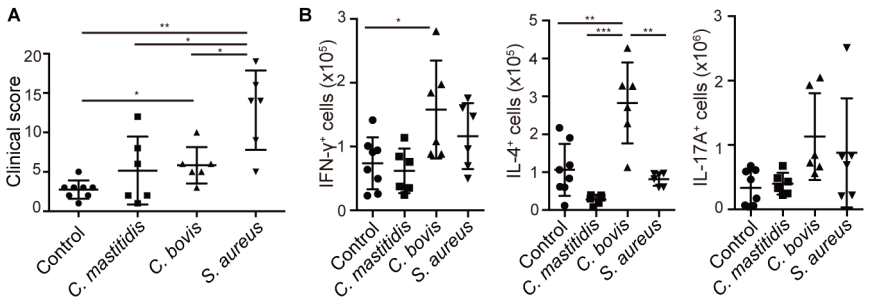

Figure 5. Contribution of *S. aureus*, *C. bovis* and *C. mastitidis* to eczematous inflammation

After withdrawal of antibiotic treatment form *Adam17*$^{\Delta Sox9}$ mice that were pretreated with antibiotics, *S. aureus*, *C. bovis* and *C. mastitidis* strains were inoculated onto the mice. See also Figure S5. (A) Clinical scores of *Adam17*$^{\Delta Sox9}$ mice after bacterial inoculation (*N*=6–8). (B) Flow cytometry analysis of IFN-γ, IL-4 and IL-17A production in CD4^{+} cells from lymph nodes of bacteria-inoculated *Adam17*$^{\Delta Sox9}$ mice (*N*=6–8). The data are shown as the mean ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001 determined by Student's t test.

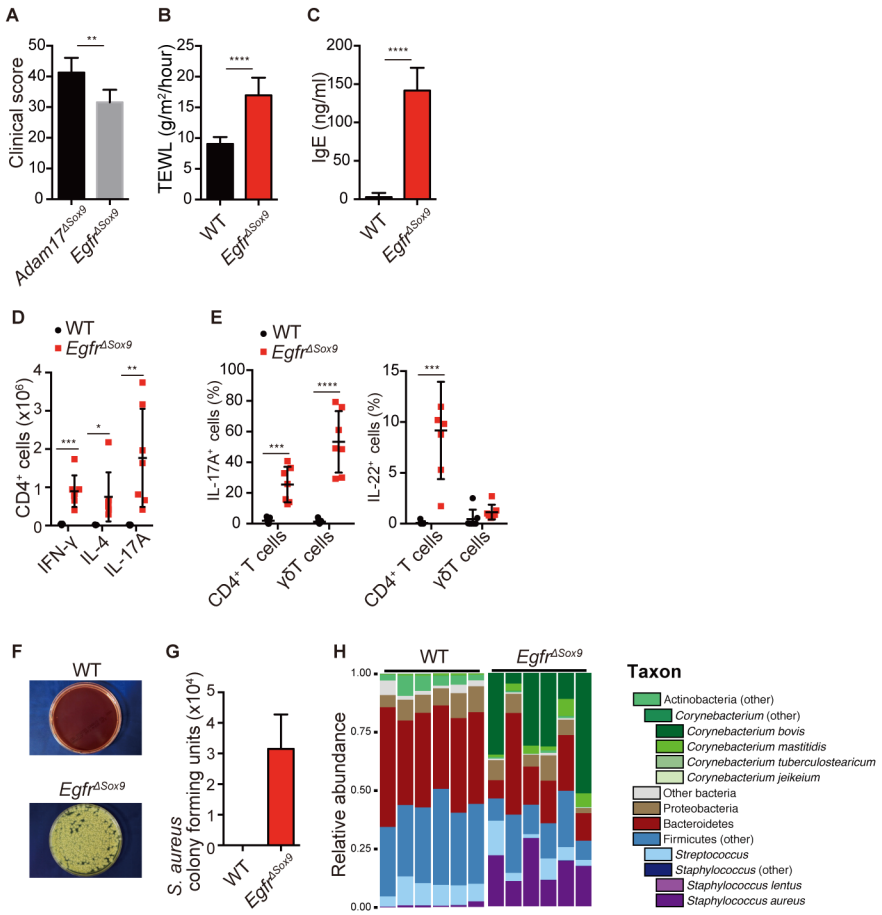

Figure 6. Ablation of EGFR-signaling induces dysbiosis and eczematous inflammation

(A) Clinical scores in *Adam17*$^{\Delta Sox9}$ and *Egfr*$^{\Delta Sox9}$ mice (*N*=7). See also Figure S6. (B, C) TEWL and serum IgE values in WT and *Egfr*$^{\Delta Sox9}$ mice (*N*=7). (D) Flow cytometry analysis of IFN-γ, IL-4 and IL-17A production in CD4^{+} cells from lymph nodes, and (E) IL-17A and IL-22 production by CD3^{+} cells in epidermis of WT and *Egfr*$^{\Delta Sox9}$ mice (*N*=7). (F, G) Quantification of *S. aureus* cultured from facial skin swab samples of WT and *Egfr*$^{\Delta Sox9}$ mice (*N*=7). (H) Microbiota of 8-week-old WT and *Egfr17*$^{\Delta Sox9}$ mice.

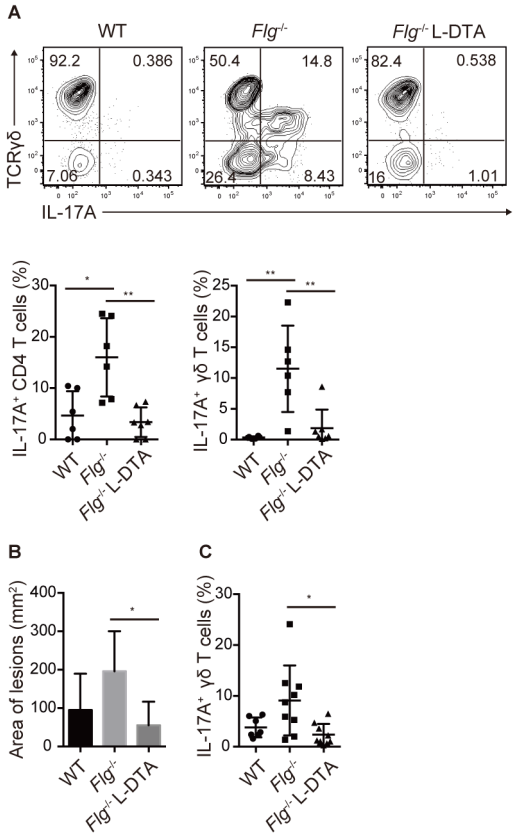

Figure 7. Langerhans cells mediate IL-17 response upon *S. aureus* inoculation

(A) Flow cytometry analysis of epidermis-infiltrating CD4 T cells and γδ T cells in topical inoculation of *S. aureus* on WT, $Flg^{-/-}$ and $Flg^{-/-}$ Langerin-DTA mice (*N*=6). (B, C) Area of inflamed lesions and epidermis-infiltrating γδ T cells of WT, $Flg^{-/-}$ and $Flg^{-/-}$ Langerin-DTA mice inoculated with *S. aureus* via occlusive dressing technique. The data are shown as the mean ± SD. **P*<0.05, ***P*<0.01 determined by Student's t test. See also Figure S7.