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Allergic skin inflammation and *S. aureus* skin colonization are mutually reinforcing.

Juan-Manuel Leyva-Castillo, PhD¹, Alexander McGurk, BA^{1,*}, Raif Geha, MD¹

¹Division of Immunology, Boston Children's Hospital, Department of Pediatrics, Harvard Medical School, Boston, MA, USA

Abstract

Atopic dermatitis (AD) lesional skin is often colonized with *S. aureus*, and the load of *S. aureus* correlates with disease severity. However, a causative and mechanistic link between *S. aureus* skin colonization and severity of AD is not well established. We made use of well-established mouse model of AD elicited by epicutaneous sensitization of tape stripped skin with ovalbumin to investigate the relationship between allergic skin inflammation and cutaneous *S. aureus* colonization. Topical application of *S. aureus* exacerbated allergic skin inflammation induced by epicutaneous sensitization with ovalbumin, whereas allergic skin inflammation generated a permissive environment for *S. aureus* persistence. Our results establish a mutually reinforcing role of allergic skin inflammation and *S. aureus* skin colonization.

1. INTRODUCCION.

Atopic dermatitis (AD) is characterized by a defective skin barrier function and a type 2 cytokine dominated local and systemic response to antigens encountered through the skin [1]. AD lesional skin is often colonized with *S. aureus*, and the load of *S. aureus* correlates with disease severity [1, 2], suggesting that the microenvironment in AD skin lesions promotes the persistence/growth of *S. aureus*, which in turn may aggravate skin inflammation. Indeed, some, but not all studies, report clinical improvement of AD following control of *S. aureus* infection [3], and a decreased AD severity following vigorous *S. aureus* decolonization of the skin [4]. However, although believed to exist, a bidirectional causative link between *S. aureus* skin colonization and AD severity has not been formally established.

We previously demonstrated that epicutaneous (EC) sensitization with ovalbumin (OVA) elicits allergic skin inflammation that shares many features with AD skin lesions [5, 6]. These include epidermal hyperplasia, infiltration with CD4⁺ T cells and eosinophils and increased local *Il4*, *Il13* and *Il17a* mRNA levels [5, 6]. In addition, OVA sensitized mice

Corresponding author: Raif S. Geha, MD. Boston Children's Hospital, Division of Immunology, One Blackfan Circle, Boston, Massachusetts 02115, USA. Phone: 617-919-2482, Fax: 617-730-0528, raif.geha@childrens.harvard.edu.

*Current address: Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, USA.

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developed a systemic response with OVA specific IgE antibodies and cytokine secretion by splenocytes in response to OVA stimulation *in vitro* [5, 6]. We made use of this well-established mouse model of AD to investigate the relationship between allergic skin inflammation and cutaneous *S. aureus* colonization.

2. MATERIAL AND METHODS.

2.1. Mice.

Il4^{-/-}, *Il13^{-/-}* and *Il4/13^{-/-}* mice on BALB/c background were previously described [7–9]. BALB/c mice were purchased from Charles River Laboratory. All mice were kept in a pathogen-free environment and fed an OVA-free diet. All procedures were performed in accordance with the Animal Care and Use Committee of the Children's Hospital Boston.

2.2. *S. aureus* preparation and quantification.

S. aureus inoculum was streaked onto tryptic soy agar plate and grown overnight at 37°C. Single colonies were picked and inoculated into a 5 ml tube containing tryptic soy broth and cultured overnight in a shaking incubator. The following morning 1:50 dilution of bacterial suspension was inoculated in 5 ml of tryptic soy broth and cultured for another 2 hrs. Bacterial concentrations were estimated by measuring absorbance at 600 nm. The bacteria were concentrated to 10⁸ CFU/50 µl of PBS, and used for cutaneous infection. CFUs were verified by overnight culturing of inoculum on Chrom-agar plates. To enumerate the bacterial load *in vivo*, *S. aureus* was labeled with PSVue794 reagent kit (LI-COR), following manufacturer's instructions. Then, PSVue794 fluorescence was quantified at different time points using Pearl® Trilogy Small Animal Imaging System (LI-COR). To enumerate the bacterial load from the skin, two 8 mm² skin biopsies were obtained. After mechanical homogenization, serial dilutions of skin homogenates were cultured on Chrom-agar plates. The growth of USA300 strain was quantified by counting only pink colonies after overnight incubation.

2.3. Epicutaneous (EC) sensitization and *S. aureus* application.

Female mice 6-8-weeks old were epicutaneously sensitized for 8 days as described previously [5, 6]. Briefly, Mice were anesthetized, and their back skin was shaved and tape-stripped with a film dressing (Tegaderm™, 3M) followed by the application of 200 µg OVA (Sigma-Aldrich) or saline every other day. At day 9, 10⁸ *S. aureus* CFU in 50 ml of PBS were superficially applied on epicutaneous sensitized skin with the help of a cotton swab. Analyses were done at D12.

2.4. Histology and measurement of epidermal thickness.

Skin specimens were fixed in 4% paraformaldehyde embedded in paraffin and H&E stained. ImageJ was used for the quantification of the epidermal thickness.

2.5. Skin cell preparation, and flow cytometry.

1cm² skin pieces from unmanipulated or tape stripped mice, were obtained. Skin pieces were finely chopped using scissors after fat removal and digested for 90 minutes in the

media containing Liberase (0.2mg/mlRoche) and DNase II (Sigma), with continuous shaking at 37° C. Digested skin homogenates were filtered, washed and resuspended in PBS and used for flow cytometry. Cells were preincubated with FcγR-specific blocking mAb (2.4G2) and washed before staining with the following monoclonal antibodies (mAbs): CD3 (17A2), CD45 (30F11), Gr1 (RB6-8C5) from eBioscience, CD11b (M1/70) from Biolegend and anti-Siglec-F (E50-2440) from BD Biosciences. Cells were analyzed by flow cytometry using an LSRFortessa machine (BD Biosciences).

2.6. mRNA expression analyses.

Total skin RNA was extracted with Total RNA Isolation Kit (Ambion). cDNA was prepared with iscript cDNA synthesis kit (Biorad). PCR reactions were run on ABI Prism 7300 (Applied Biosystems) sequence detection system platform. Taqman primers and probes were obtained from Life technologies. The housekeeping gene β₂-microglobulin was used as an internal control. Relative mRNA expression was quantified using the 2⁻ Ct method.

2.7. OVA-specific IgE measurement.

anti-OVA IgE concentrations were measured in sera collected at day 12 by means of ELISA, using a homemade sandwich ELISA for anti-OVA IgE. The capture detection antibody (rat anti-mouse IgE clone R35-72) was obtained from BD Bioscience and purified mouse anti-OVA IgE (clone TOe) standard antibody was produced in the lab. OVA was biotinylated using a kit (Pierce) and used for detection.

2.8. Cell culture and in vitro cytokine expression.

Single cell suspensions of splenocytes were cultured and stimulated with OVA and their supernatants analyzed for cytokines by ELISA as previously described [10].

2.9. Statistical analysis.

Statistical significance was determined by the two-tailed Student's t test. A p value <0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION.

3.1. Application of *S. aureus* exacerbates allergic skin inflammation caused by EC sensitization with OVA.

Shaved skin of wild-type (WT) Balb/c mice was tape stripped followed by topical application of OVA or saline, with or without subsequent application of *S. aureus* (Fig. 1A). As previously reported [5, 6], EC sensitization with OVA caused epidermal thickening, accumulation of CD3⁺ T cells and eosinophils, and local upregulation of *Il4*, *Il13*, and *Il17a*, but not *Ifng* expression (Fig. 1 B–D and data not shown), compared to EC sensitization with saline. In addition, mice EC sensitized with OVA had serum OVA specific IgE antibodies (Fig. 1E), and their splenocytes secreted IL-4, IL-13, and IL-17A in response to *in vitro* restimulation with OVA (Fig. 1F). Topical application of 1×10⁸ CFUs of methicillin resistant *S. aureus* (MRSA) strain SF8300 (USA300) on skin EC sensitized with OVA significantly increased the local accumulation of T cells and eosinophils (Fig. 1B–C), as well as *Il4* and

Il13 expression compared to EC sensitized skin with OVA (Fig. 1D). *S. aureus* application also abolished *Il17a* upregulation caused by OVA sensitization (Fig. 1D), possibly due to downregulation of *Il17a* expression by the increased Th2 cytokines. The effects of *S. aureus* on cytokine expression at sites of cutaneous antigen sensitization application was not due to an enhanced systemic response to EC sensitization, as there was no detectable effect on serum levels of OVA-specific IgE or cytokine secretion by splenocytes in response to *in vitro* restimulation with OVA (Fig 1E–F). Application of *S. aureus* to skin EC sensitized with saline caused increased cutaneous accumulation of CD3⁺ T cells, but had no detectable effect on eosinophil accumulation or local expression of *Il4*, *Il13* and *Il17a*. Of note *S. aureus* application to saline sensitized skin causes cutaneous upregulation of *Il17a* on D9 and D10 that wanes by D12 (data not shown and Fig.1D). Altogether the above results indicate that *S. aureus* colonization aggravates allergic skin inflammation.

3.2. IL-4 and IL-13 impair *S. aureus* clearance from sites of allergic skin inflammation.

Type 2 cytokines promote *S. aureus* skin infection by suppressing the production of antimicrobial peptides (AMPs) by keratinocytes, impairing skin barrier integrity and increasing adherence of *S. aureus* to the skin [11]. In contrast, IL-17A promotes the clearance of *S. aureus* from skin by inducing production AMPs and neutrophil chemoattractant chemokines by keratinocytes [12]. In a previous study, we reported that antigen sensitization renders the skin of flaky tail mice susceptible to *S. aureus* skin colonization [13]. However, these mice have mutations in both *filaggrin* and *ma* (matted hair) genes, and their skin demonstrates type 17 cytokine dominated inflammation, which is not the case in either *Flg*^{-/-} mice or patients with AD. To analyze whether type 2 cytokine dominated allergic skin inflammation promotes *S. aureus* skin colonization, mice were EC sensitized with OVA or saline for 8 days and PSVue 794 labeled *S. aureus* was applied the following day according to the protocol in Fig. 1A. *S. aureus* load was examined by *in vivo* whole animal imaging system, as well as by measuring the numbers of colony forming units (CFUs) in skin homogenates plated on Chromagar. *In vivo* imaging revealed an increase in PSVue 794 fluorescence in mice EC sensitized with OVA compared to saline sensitized controls (Fig. 2A). Moreover, significantly higher numbers of CFUs were recovered from homogenates of OVA sensitized skin compared to saline sensitized skin (Fig. 2B). To investigate the role of type 2 cytokines in *S. aureus* colonization at sites of allergic inflammation, we made use of *Il4*^{-/-}, *Il13*^{-/-} and *Il4/Il13*^{-/-} mice. Topical *S. aureus* application to OVA-EC sensitized skin of WT, *Il4*^{-/-}, *Il13*^{-/-} and *Il4/Il13*^{-/-} mice promoted similar epidermal hyperplasia (Fig. 2C). Accumulation of eosinophils, but not T cells, was decreased in OVA sensitized skin of *Il4*^{-/-}, *Il13*^{-/-} and *Il4/Il13*^{-/-} mice compared with WT controls (Fig. 2D). Moreover, *Il4*^{-/-}, *Il13*^{-/-} and *Il4/Il13*^{-/-} mice exhibited a significantly increase in *Il17a* mRNA expression compared with WT controls (Fig. 2E), and importantly, demonstrated a significantly decreased *S. aureus* load in OVA sensitized skin compared with WT control (Fig. 2F). Our results indicate that the type 2 cytokines IL-4 and IL-13 promote *S. aureus* persistence at sites of allergic skin inflammation. This effect could be mediated by suppressing local IL-17A production.

Our results establish a mutually reinforcing role of allergic skin inflammation and *S. aureus* skin colonization. Furthermore, they provide a mechanistic explanation for the recent

observation that clinical improvement and amelioration of Th2 biomarkers in AD patients following IL-4R α blockade correlate with reduced abundance of *S. aureus* in the skin [14].

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ABBREVIATIONS

| | |
|------------|-------------------|
| AD | Atopic dermatitis |
| EC | epicutaneous |
| OVA | ovalbumin |

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Highlights.

- Topical application of *S aureus* exacerbated allergic skin inflammation.
- Allergic skin inflammation generated a permissive environment for *S. aureus* persistence.
- IL-4 and IL-13 impair *S. aureus* clearance from sites of allergic skin inflammation.

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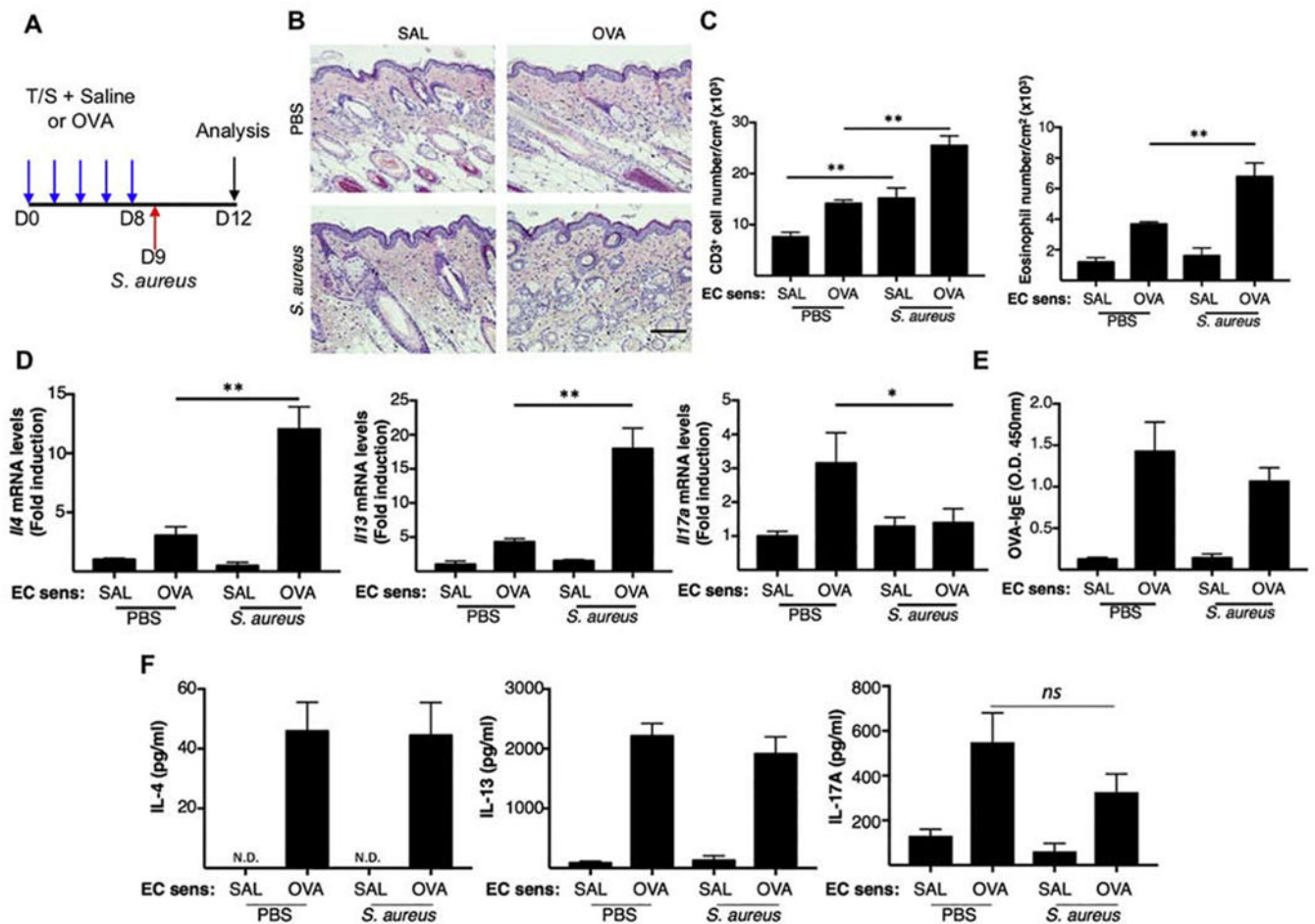


Figure 1. Application of *S. aureus* exacerbates allergic skin inflammation caused by EC sensitization with OVA.

A. Experimental protocol. Mice were EC sensitized mice with OVA or saline on tape stripped skin with or without application of *S. aureus*. **B.** Representative H&E sections. Scale bar: 100 μ m. **C.** Skin CD3⁺ T cell (left) and eosinophil (right) numbers. **D.** Skin *Il4*, *Il13* and *Il17a* mRNA levels **E.** OVA-specific IgE. **F.** Cytokine production by OVA stimulated splenocytes. Results in A-E are representative of 2 independent experiments with 4-5 mice/group. Bars represent means \pm SEM. * = $p < 0.05$, ** = $p < 0.005$. Significance was calculated by using two-tailed Student's t test.

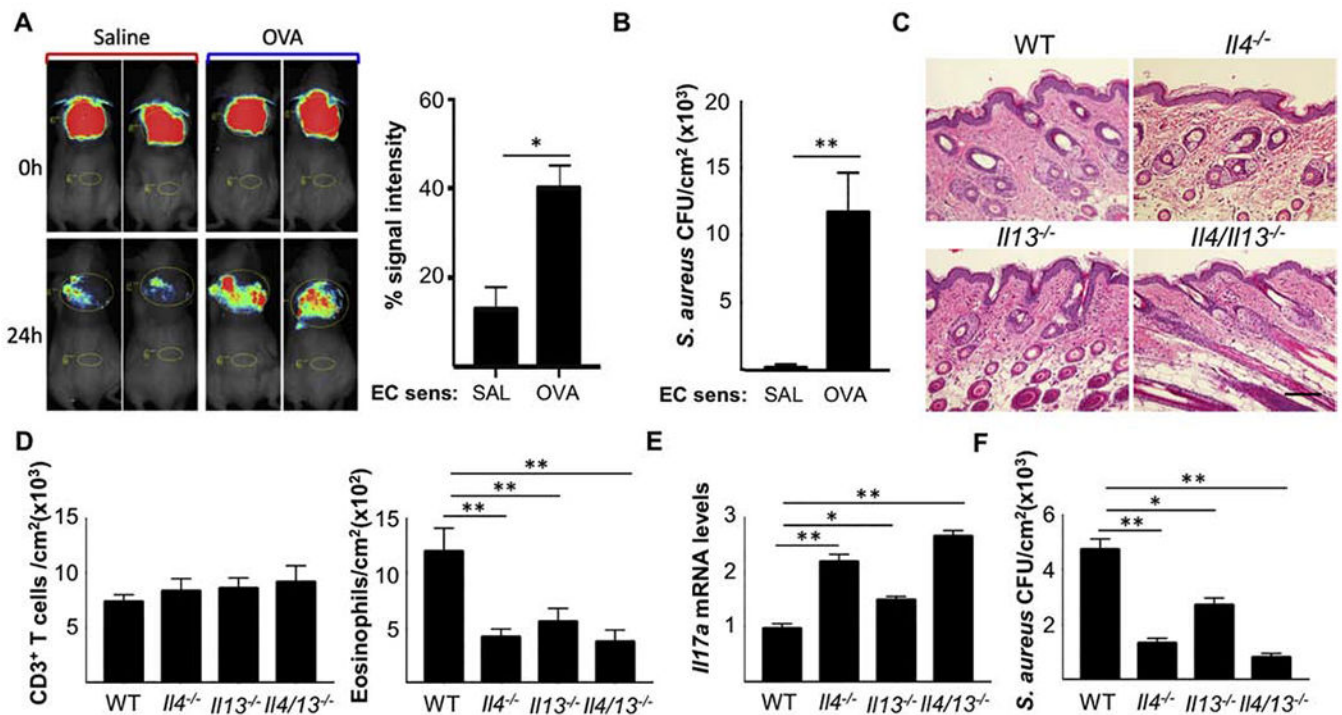


Figure 2. IL-4 and IL-13 impair *S. aureus* clearance from sites of allergic skin inflammation.

A. Representative *in vivo* imaging (left) and quantitation (right) of *S. aureus* fluorescence. **B.**

S. aureus CFUs in skin homogenates. **C.** Representative H&E sections. Scale bar: 100 mm.

D. Numbers of CD3⁺ T cells (left) and eosinophils (right) in skin. **E.** Skin *Il17a* mRNA

levels **F.** *S. aureus* CFUs in the OVA sensitized skin of *Il4*^{-/-}, *Il13*^{-/-} and *Il4/Il13*^{-/-} mice and

WT controls. Results in B-F are representative of 2 independent experiments with 4-5 mice/

group. Bars represent means±SEM. * = p<0.05, ** =p<0.005. Significance was calculated

by using two-tailed Student's t test.