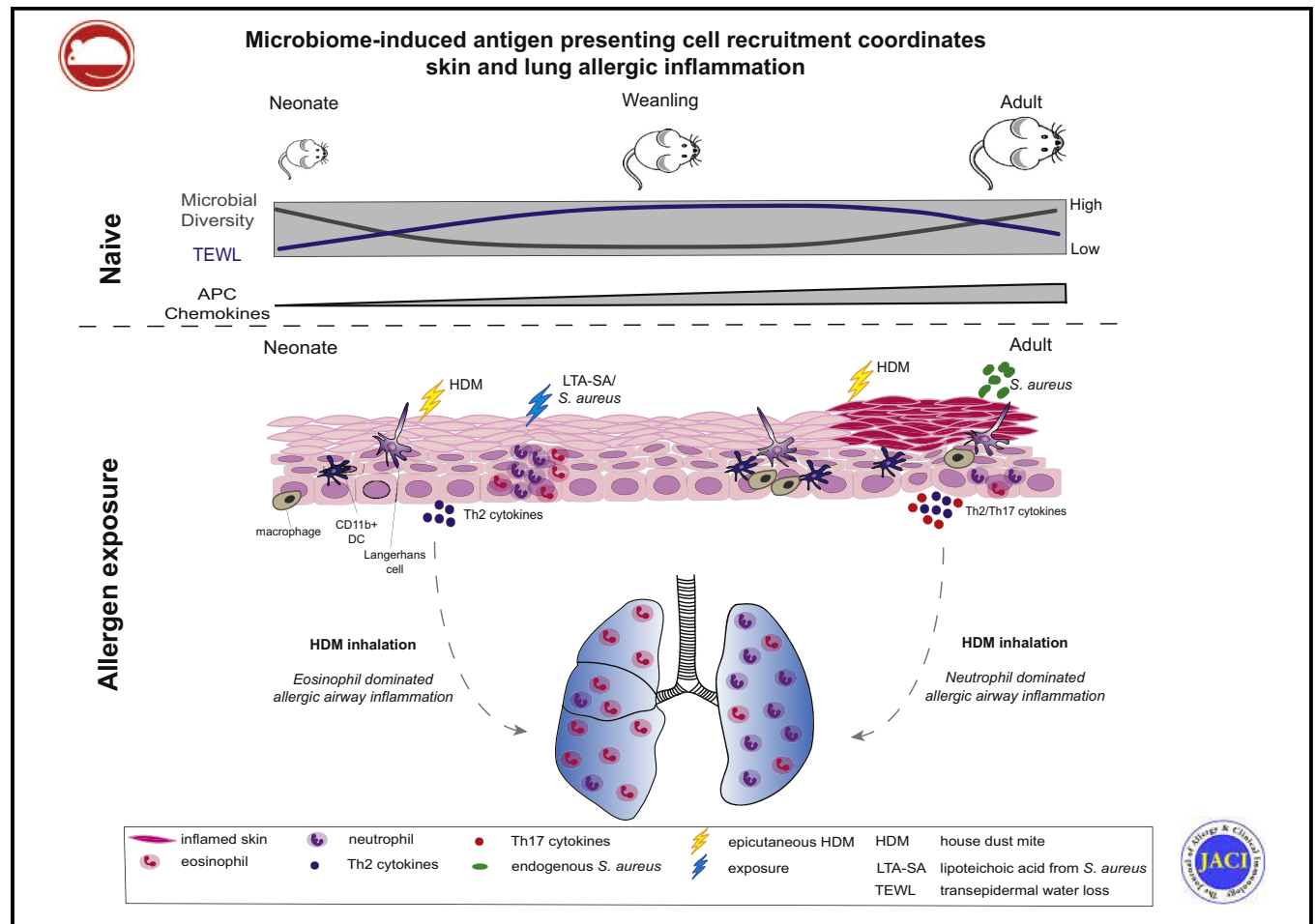


Microbiome-induced antigen-presenting cell recruitment coordinates skin and lung allergic inflammation

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GRAPHICAL ABSTRACT



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Background: Allergic skin inflammation often presents in early childhood; however, little is known about the events leading to its initiation and whether it is transient or long-term in nature.

Objective: We sought to determine the immunologic rules that govern skin inflammation in early life.

Methods: Neonatal and adult mice were epicutaneously sensitized with allergen followed by airway allergen challenge. Epicutaneous application of labeled allergen allowed for determination of antigen uptake and processing by antigen-presenting cells. RNAseq and microbiome analysis was performed on skin from neonatal and adult specific pathogen-free and germ-free mice.

Results: A mixed T_H2/T_H17 inflammatory response in the skin and the lungs of adult mice was observed following sensitization and challenge. Comparatively, neonatal mice did not develop overt skin inflammation, but exhibited systemic release of IL-17a and a T_H2 -dominated lung response. Mechanical skin barrier disruption was not sufficient to drive allergic skin inflammation, although it did promote systemic immune priming. Skin of neonatal mice and adult germ-free mice was seeded with low numbers of antigen-presenting cells and impaired chemokine and alarmin production. Enhanced chemokine and alarmin production, and seeding of the skin with antigen-presenting cells capable of instructing recruited cells to elicit their effector function, was, at least in part, dependent on formation of the microbiome, and consequently contributed to the development of overt skin disease.

Conclusions: These data shed light on the principles that underlie allergic inflammation in different tissues and highlight a window of opportunity that might exist for early-life prevention of allergic diseases. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: Epicutaneous sensitization, early life, allergy, immune maturation, airway inflammation

Immediately following birth, newborns are exposed to environmental factors including microbes and allergens. These exposures occur concomitantly with, and have an impact on, the maturing immune system, with consequences for disease susceptibility and development.^{1,2} Microbial exposure plays a fundamental role in the development of immune homeostasis in the lung and gut.³ Alterations in microbial composition, or depletion of the microbiota, can lead to perturbations in immune development and thereby an increased susceptibility to, or exaggeration of, allergic diseases.

The skin provides a physical barrier to the environment, and the outer layer, the epidermis, is formed by terminally differentiated keratinocytes and lipids.⁴ Alterations in epidermal differentiation and lipid composition can lead to impairment of skin barrier function, which enhances permeability and penetration of environmental factors, such as allergens.⁵ Antigen-presenting cells (APCs) in the skin transport antigens to the draining lymph nodes where they prime T cells, and in addition, are crucial for the reactivation of effector T cells in the skin tissue itself, such that the T cells can exert their effector function at that site. Langerhans cells (LCs), which reside in the epidermis, were long thought to be the main APC type in the skin. However, it has become evident that

Abbreviations used

AD:	Atopic dermatitis
APC:	Antigen-presenting cell
cDC:	Conventional dendritic cell
GF:	Germ free
GO:	Gene ontology
LC:	Langerhans cell
LTA-SA:	Lipoteichoic acid from <i>Staphylococcus aureus</i>
OVA:	Ovalbumin
SPF:	Specific pathogen-free
TEWL:	Transepidermal water loss
Treg:	Regulatory T

other APCs such as macrophages and conventional dendritic cells (cDCs), cDC1 and cDC2, reside in the dermis and can present antigen to local T cells.⁶

Sensitization to allergens via the skin can lead to the development of allergic skin inflammation or atopic dermatitis (AD). The onset of AD occurs predominantly in childhood. For most children, this inflammation is transient and they “outgrow” it; however, in a minority of cases, the disease persists into adolescence and adulthood.^{7,8} Although it has been suggested that the age of disease onset and the severity of the disease play a part in determining whether allergic skin inflammation persists into adulthood, the parameters that define whether the disease becomes persistent, or not, remain unknown.

Here, we set out to determine the immunologic rules that govern skin inflammation in early life using an experimental model of neonatal and adult epicutaneous allergen sensitization. We found that the nature and severity of allergic skin inflammation change with age. Skin allergen sensitization followed by allergen challenge in the lungs induced a mixed T_H2/T_H17 inflammatory response in the skin and the lung of adult mice, whereas neonatal mice exhibited a T_H2 -dominated inflammatory response in the lung and no overt skin inflammation. Analysis of naive skin tissue revealed that until the skin microbiome had formed, the skin exhibits impaired chemokine and alarmin production and low numbers of skin-resident APCs. Formation of the microbiome drives chemokine production, seeding of the tissue with APCs, and consequent immune maturation over time. Following this maturation process, mature cutaneous APCs are able to localize inflammation to the site of exposure, the skin, and cause overt allergic skin inflammation. Together, these results indicate that early-life cutaneous immune maturation is, at least in part, dependent on microbial exposure, and manifestation of disease is determined by the maturation state of the barrier tissue.

METHODS

Experimental animals

Male and female BALB/c mice were purchased from Charles River Laboratories (L'Arbresle Cedex, France) and housed under SPF conditions and time-mated to obtain offspring. The date of birth was recorded as day 0 of life, and both male and female mice were used for all experiments. Male and female germ-free (GF) BALB/c mice were obtained from the Clean Mouse Facility, University of Bern, Bern, Switzerland. All animal experiments were performed in accordance with institutional guidelines and Swiss federal and cantonal laws on animal protection.

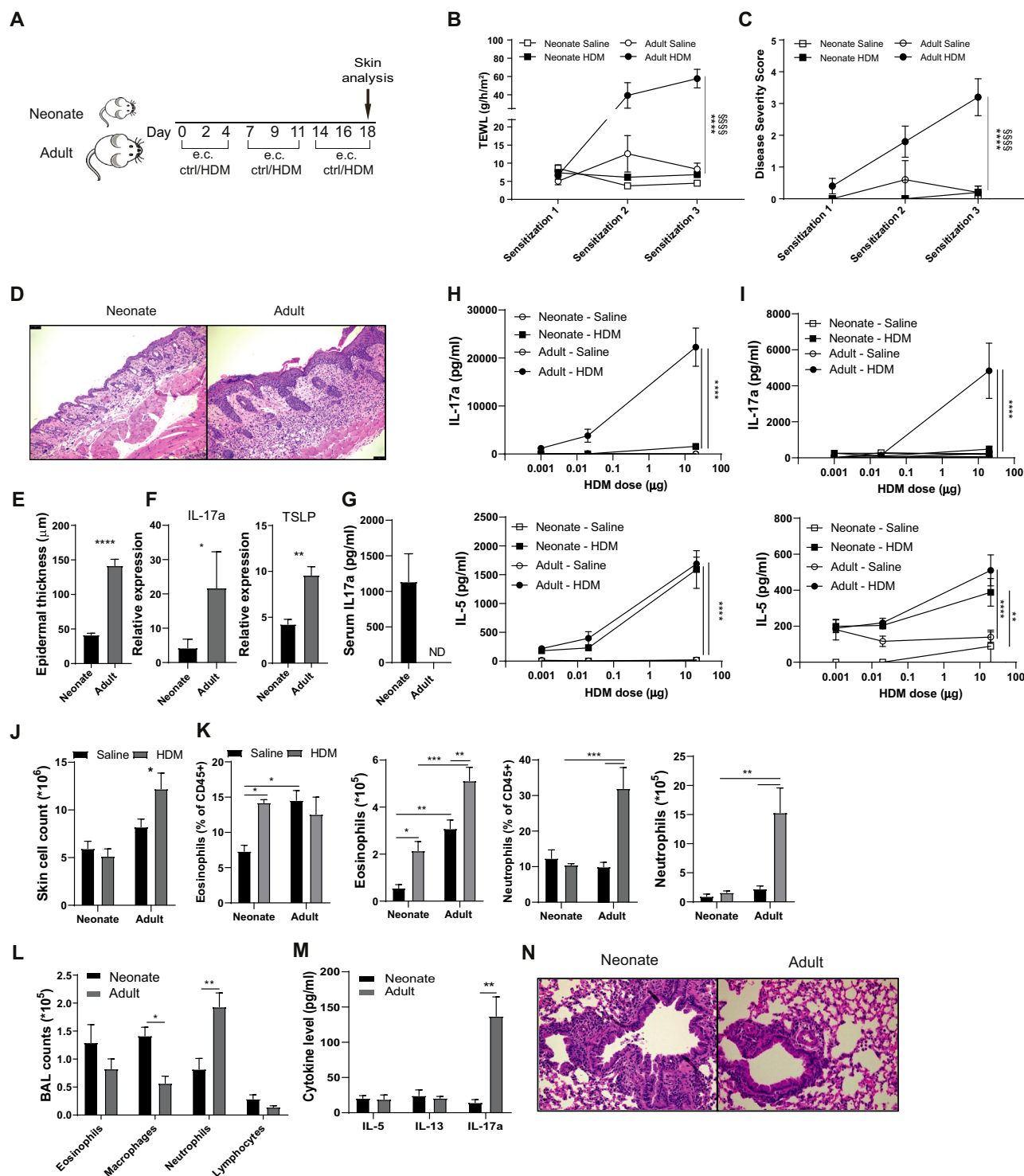


FIG 1. Age of first epicutaneous allergen sensitization determines nature and severity of allergic skin inflammation. **A**, Experimental model. **B**, TEWL. **C**, Disease severity score. **D**, Representative H&E-stained skin sections; 100× magnification. **E**, Quantification of epidermal thickness. **F**, Cutaneous IL-17a and TSLP mRNA expression levels. **G**, Serum IL-17a levels. **H** and **I**, IL-5 and IL-17a cytokine levels in supernatant of HDM-stimulated cells isolated from (Fig 1, **H**) skin-draining lymph nodes and (Fig 1, **I**) inguinal lymph nodes. **J** Total skin cell counts and **K** eosinophil and neutrophil frequencies (of CD45⁺ cells) and absolute numbers. **L**, Differential bronchoalveolar lavage (BAL) cell counts. **M**, BAL cytokine levels. **N**, Representative H&E-stained lung sections; 200× magnification. All results representative of data generated in 2 different experiments. All data are expressed as mean $\pm$ SEM ($n = 5$ mice for each group [Fig 1, **A** and **B**, **L-M**], $n = 4$ for neonates and $n = 5$ for adults [Fig 1, **E-K**]). Statistical significance was determined with 2-way ANOVA test, except for Figure 1, **E** and **F**, in which unpaired Student *t* test with Welch correction was used. *Ctrl*, Control; *e.c.*, epicutaneous; *ND*, not detectable. * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$ compared with neonatal (HDM) condition. §§§§ $P \leq .0001$ compared with adult saline condition.

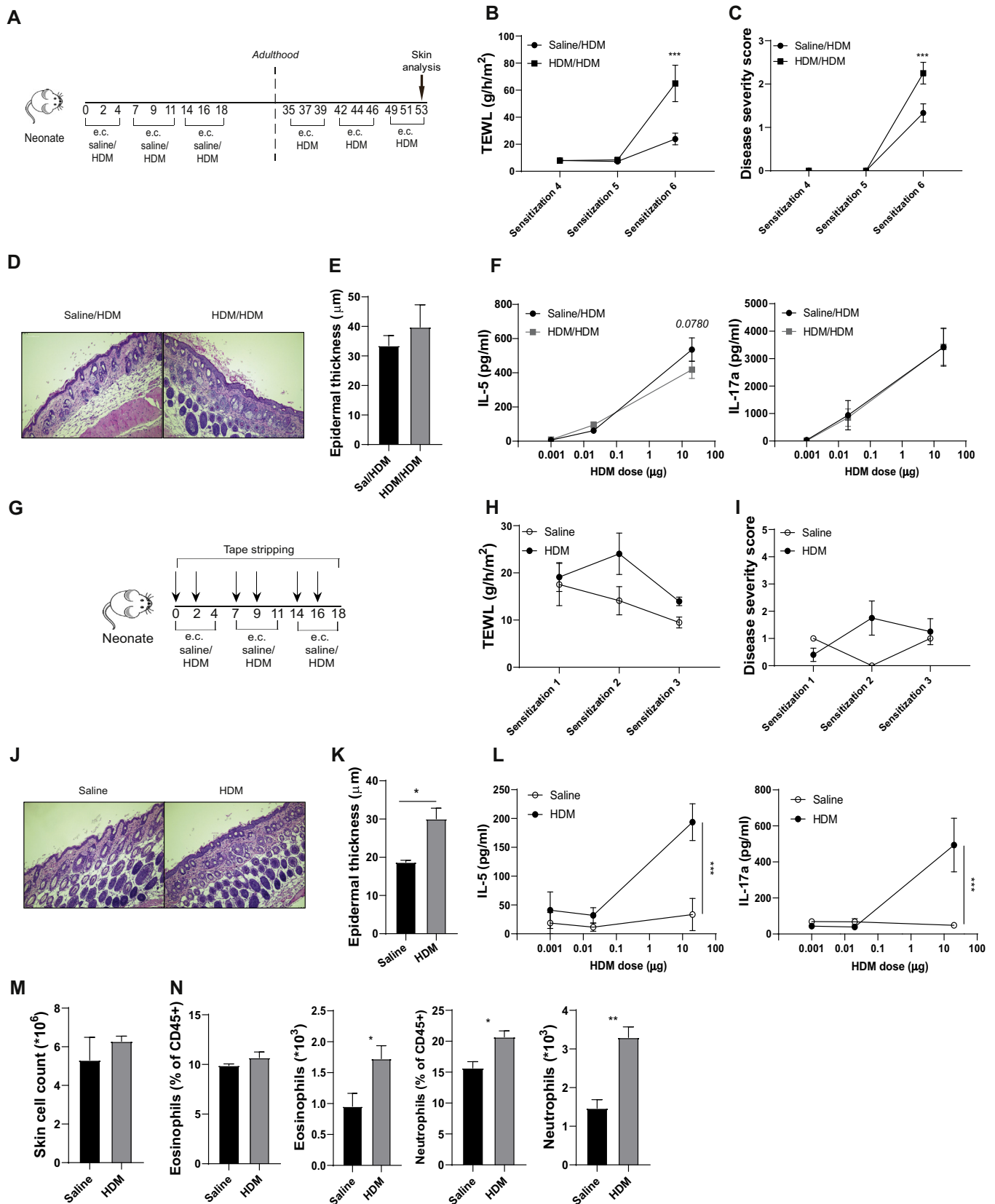


FIG 2. Limited overt skin inflammation in neonates is not due to lack of skin barrier impairment. **A**, Experimental model. **B**, TEWL. **C**, Disease severity score. **D**, Representative H&E-stained skin sections; 100× magnification. **E**, Quantification of epidermal thickness. **F**, IL-5 and IL-17a cytokine levels in supernatant of HDM-stimulated cells isolated from skin-draining lymph nodes. **G**, Experimental model. **H**, TEWL.

Animal model of the atopic march

First-generation BALB/c (Charles River Laboratories) neonates (7–10 days old) or adult (6-week-old) mice were shaved on the dorsal skin area. Epicutaneous sensitization with 15 μ g house-dust mite (HDM) extract (Greer Laboratories Inc, Lenoir, NC) in saline or saline control was performed by application of gauze (Dermaplast, Hartmann Group, Heidenheim, Germany) on the dorsal skin area, which was secured by a bioclusive dressing (Systagenix, Gatwick, UK) and covered with adhesive tape (Mölnlycke Health Care, Gothenburg, Sweden) to prevent removal of the gauze and consequently HDM ingestion. The sensitization was performed for 3 consecutive weeks, each week consisting of 5 consecutive days of sensitization, with a change of the gauze on day 3 and removal of the gauze on day 5, followed by a 2-day recovery period. Measurements were taken on the last day of the third sensitization round or after each round of sensitization depending on the experimental setting. Tape stripping was performed with transparent tape (Scotch, 3M, St Paul, Minn) on dorsal skin area after shaving (10 repeats total) before application of allergen during each sensitization round. Lipoteichoic acid from *Staphylococcus aureus* (LTA-SA) (Invivogen, San Diego, Calif) (25 μ g) or live *S aureus* (Newman strain [1.1×10^9 colony forming units [CFU]; range, 9.2×10^8 to 1.3×10^9]) in 100 μ L was applied topically on the dorsal skin area before allergen sensitization during each sensitization round. Skin microbiome was depleted by topical application of Neosporin crème (bacitracin, neomycin, and polymyxin) (Johnson and Johnson, New Brunswick, NJ) or control petrolatum crème (Vaseline, Unilever, London, UK) on the dorsal skin area 3 times per week during each sensitization round.

Transepidermal water loss measurement

Transepidermal water loss (TEWL) measurement was performed on naive skin or 4 hours after the end of each allergen sensitization round on the sensitized dorsal skin area using a TEWA meter TM 300 (C.K. Electronic GmbH, Köln, Germany). Two measurements were taken per mouse.

Skin disease severity scoring

Dorsal skin was evaluated for dryness, rash, excoriation, baldness, and lichenification at the end of each sensitization round on the sensitized dorsal skin area. Each sign was scored with a 1 = present or 0 = absent; if more than 2 excoriations, this was given a score of 2. The disease severity score reflects the sum of this scoring.

Histological analysis of skin tissue

Dorsal skin tissue from the sensitized area was excised, fixed in formalin solution, and embedded in paraffin. Tissue sections (4 μ M) were stained with hematoxylin and eosin. Epidermal thickness measurements were performed on skin sections (100 $\times$ magnification) using ImageJ software (NIH, Bethesda, Md).⁹

Flow cytometry

Characterization of APC populations in the skin was performed by flow cytometry. Skin tissue was excised and intradermally injected with liberase working solution (25 mg/mL Liberase TL [Roche, Mannheim, Germany] and 10 mg/mL DNase I [Sigma Aldrich, St Louis, Mo] in RPMI1640 medium containing penicillin/streptomycin [Invitrogen, Grand Island, NY], HEPES [BioConcept, Allschwil, Switzerland], and β -mercaptoethanol [Thermo Fischer Scientific, Waltham, Mass]), and incubated for 2 hours at 37°C.

Samples were then dissociated, filtered through a 100- μ m and 70- μ m cell strainer using RPMI medium containing 5% FCS, washed, and resuspended in PBS supplemented with 0.2% BSA. Total cell counts were determined using a Coulter Counter. Cells were then stained for flow cytometry with Zombie red fixable viability stain-PE-Texas red (423109, 1:2000 dilution), CD45-Fluorescein isothiocyanate (FITC) (109805, 1:800 dilution), I-A/I-E-Alexa Fluor 700 (AF700) (107622, 1:600 dilution), CD64-phycoerythrin (PE) (BD Biosciences, San Jose, Calif; 558455, 1:800 dilution), CD11c-Allophycocyanin Cy7 (APC-Cy7) (117324, 1:800 dilution), CD11b-Allophycocyanin (APC) (101212, 1:800 dilution), CD24-Phycoerythrin Cy7 (Pe-Cy7) (101812, 1:800 dilution), Ly6c-Pacific Blue (PB) (128014, 1:600 dilution), CD103-Peridinin chlorophyll protein-Cy5.5 (PerCP/Cy5.5) (121416, 1:600 dilution), CCR2-Brilliant violet 650 (BV650) (BD Biosciences, 747968, 1:400 dilution), SiglecF-Alexa Fluor 647 (AF647) (BD Biosciences, 562680, 1:400 dilution), Ly6g-biotin (127604, 1:400 dilution), and Streptavidin-Phycoerythrin Cy7 (Pe-Cy7) (405206, 1:400 dilution). If not indicated otherwise, antibodies were purchased from Biolegend (San Diego, Calif). Fluorescence minus one internal staining controls were set up by pooling cells from all the different experimental groups. Cells were acquired on an LSR-II (BD Biosciences) flow cytometer. Samples were analyzed using FlowJo 10.2 software (Tree Star Inc, Ashland, Ore) on the basis of gating strategies as provided in Fig E2 in this article's Online Repository at www.jacionline.org.

Antigen uptake and processing

Dorsal skin of naive neonatal and adult mice was shaved and 100 μ g FITC-labeled or DQ-labeled ovalbumin (OVA) (Invitrogen) (in 50 μ L total volume) was applied topically. DQ-OVA acquires a fluorescent signal that can be detected in the FITC channel after being processed by APCs. The presence of FITC-OVA or DQ-OVA-positive APCs in the skin was determined by flow cytometry at 4 hours and 24 hours after application.

Skin gene expression analysis

Relative skin mRNA expression was assessed by quantitative RT-PCR using the following primer sets: *TSLP* forward 5'-TGA CAA GCA ATG AGA CGATGA G-3' and reverse 5'-CCC ACG GAC AGT TTG ATT CTT C-3'; *IL-5* forward 5'-TGA CAA GCA ATG AGA CGA TGA G -3' and reverse 5'-CCCACGGACAGTTTGATTCTT C -3'; *IL-17a* forward 5'-ACCCTG GACTCTCCACCGCAA -3' and reverse 5'-GGTGGTCCAGCTTTCCC TCCG-3'; *IL-33* forward 5'-GCTGCGTCTGTTGACACATT-3' and reverse 5'-CACCTGGTCTTGCTCTTGGT-3'; *CCL8* forward 5'-AGCTGTGGTT TTCCAGACCAA-3' and reverse 5'-GTGTGAAGGTTCAAGGCTGC-3'; *CCL9* forward 5'-GCCCAGATCACACATGCAAC-3' and reverse 5'-AGGA CAGGCAGCAATCTG AA-3'; *CCL11* forward 5'-TCA CTT CCT TCA CCT CCC AG-3' and reverse 5'-CTA TGG CTT TCA GGG TGC AT-3'; and *GAPDH* forward 5'-GGG TGT GAA CCA CGA GAA AT-3' and reverse 5'-CCT TCC ACA ATG CCA AAG TT-3'. Ten-microliter PCR reactions were set up containing 2 μ L of template cDNA at a concentration of 50 μ g/mL, 5 μ L SsoAdvanced SYBR green reaction mix (Bio-Rad, Hercules, Calif), 0.25 μ L of each primer at a concentration of 20 μ M, and 2.5 μ L nuclease-free water. All reactions were performed in duplicate, and each gene was normalized to GAPDH. Quantitative PCR was performed on the CFX96 Touch Real Time PCR Detection System (Bio-Rad) using the following conditions: 1 cycle at 95°C for 2 minutes and then 40 cycles at 95°C for 15 seconds and 60°C for 30 seconds, followed by a dissociation stage at 65°C for 31 seconds and cycles of 5 seconds starting at 65°C, raising 0.5°C per cycle, to obtain melting

I, Disease severity score. J, Representative H&E-stained skin sections; 100 $\times$ magnification. K, Quantification of epidermal thickness. L, IL-5 and IL-17a cytokine levels in supernatant of HDM-stimulated cells isolated from skin-draining lymph nodes. (M) Total skin cell numbers and (N) eosinophil and neutrophil frequencies (of CD45⁺ cells) and absolute numbers. All results are representative of data generated in 2 different experiments. All data are expressed as mean $\pm$ SEM (n = 6 mice for each group [Fig 2, A–F], n = 4 mice for saline and n = 5 for HDM group [Fig 2, G–M]). Statistical significance was determined with 2-way ANOVA test, except for Fig 2, E, K, M, and N, in which unpaired Student t test with Welch correction was used. e.c., Epicutaneous; Sal, saline. * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$ compared with neonatal (HDM) condition.

curves for specificity analysis. Following amplification, C_q values were obtained using the CFX Manager™ software 2.1 (Bio-Rad).

Lymph node restimulation and cytokine analysis

Cells (4×10^5) from skin-draining lymph nodes (axillary and brachial) or inguinal lymph nodes were plated and stimulated for 72 hours with different concentrations of HDM extract (Greer Laboratories Inc, Lenoir, NC). Supernatants were harvested after 72 hours to quantify cytokine secretion. IL-5 and IL-17a levels were quantified in cell culture supernatants using a Ready-SET-Go ELISA kit for each cytokine (Invitrogen). Assays were performed according to manufacturer's instructions.

RNA sequencing and data processing

Total skin RNA was converted into cDNA libraries using the Illumina TruSeq RNA Sample Preparation Kit (Illumina) according to the protocol supplied by the manufacturer and submitted to high-throughput sequencing using the Illumina HiSeq 2500 (Illumina) system. Sequencing data were processed using the Illumina Pipeline Software version 1.82.

Raw fastq files were processed using RNASik v1.4.7 with default settings.¹⁰ RNASik wraps alignment, QC metrics, and quantification in the 1 script, giving a gene-annotated counts table for differential expression analysis. Alignment was performed using STAR v2.5.2¹¹ against the iGenomes (http://sapac.support.illumina.com/sequencing/sequencing_software/igenome.html) GRCh38 reference with the associated gene-model GTF. featureCounts¹² quantified read counts to annotated genes. Visualization and analysis of differentially expressed genes was performed using Degust v3.1.0 (<http://degust.erc.monash.edu/>).

Gene ontology analysis

Gene ontology (GO) enrichment analysis was performed using Gorilla (Gene Ontology enrichment analysis and visualization tool).¹³ Differentially expressed genes used for GO analysis were selected on the basis of false detection rate more than 0.05 and log fold change more than 2. A list of all genes analyzed in these samples was used as the background list in Gorilla.

Bacterial DNA isolation from skin swabs

Skin swabs were collected by swabbing the dorsal skin area of a mouse for 20 seconds using an Eswab (Copan) and processed as previously described.¹⁴ Swabs were stored at -80°C before DNA extraction. Eswab liquid was centrifuged for 10 minutes at 14,000g. Pellets were incubated with 9000 U of Ready-Lyse Lysozyme (Epicentre) for 1 hour at 37°C before incubation with 20 μL proteinase K in 200 μL AL buffer (QIAamp DNA Mini Kit) for 30 minutes at 56°C with shaking. Resulting lysates were further processed according to the manufacturer's protocol with 2 additional washing steps. DNA was eluted in 50 μL water (Sigma Aldrich) by centrifugation at 6000g for 1 minute. All processing steps were carried out in bacterial DNA-free conditions under a laminar flow hood.

16S rRNA library preparation and sequencing

16S rRNA gene amplicon library was generated using 27F and 338R universal primers targeting the V1-V2 hypervariable region of the 16S rRNA gene as previously described.¹⁵ Briefly, primers were designed as follows: 27F-5'-**TGATACGGCGACCACCGAGATCTACACTATGGTAATTC**CAG MGTTYGATYMTGGCTCAG-3' and 338R-5'-**CAAGCAGAAGACGG**CATACGAGATNNNNNNNNNNNAGTCAGTCAGAGCTGCCTCCCG TAGGAGT-3' where Illumina adaptor sequences are bold, linkers are italicized, and NNNNNNNNNNNN sequences represent the sample-specific MID tag barcodes. Each PCR reaction was performed in duplicate in 20 μL total volume using the AccuPrime Taq DNA polymerase high-fidelity kit (Invitrogen) with 4 μL of DNA template and 0.44 μL of each 27F and 338R primers at 10 mM. Amplification conditions consisted of 3 minutes of initial

denaturation at 94°C , followed by 35 cycles of 30 seconds of denaturation at 94°C , 30 seconds of annealing at 56°C , and 90 seconds of extension with 5 minutes of final extension at 72°C . Amplicons size and quantity was assessed on the LabChip GX (Perkin Elmer, Waltham, Mass) before pooling PCR products in equimolar amounts of amplicons. Pooled library was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, Calif), and sequencing was performed on an Illumina MiSeq platform with MiSeq reagent kit V2-500 (pair-end, 2x250).

16S rRNA sequencing data analysis

16S rRNA sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME, v1.9.0) software¹⁶ as described previously.¹⁷ Merging of paired forward and reverse reads was performed using fastq-join before demultiplexing and quality filtering (quality Phred score $Q > 20$, < 3 low-quality base calls). Operational taxonomic units (OTUs) were assigned using a closed OTU picking strategy with Uclust at 97% identity against the 97% Greengenes reference database (v13.5).¹⁸ Low abundance OTUs with at least 3 counts in 2 samples were removed as well as plant-contaminant OTUs (Chloroplast class). Visualization and analysis of OTU tables was performed using Genocrunch (v2) (<https://genocrunch.epfl.ch/>).

Statistical analysis

Student *t* test (unpaired, 2-tailed, with Welch correction) or 2-way ANOVA with repeated measures was used to calculate significance levels between different groups. Statistical tests used for microbiota analysis are indicated in the "16S rRNA Sequencing Data Analysis." Statistical parameters are reported in each figure legend. *n* represents the number of biological replicates. Graph generation and statistical analyses were performed using Prism software, version 7.0c (GraphPad, La Jolla, Calif).

RESULTS

The nature and severity of allergic skin inflammation is dependent on the age of first skin sensitization

The onset of allergic skin inflammation typically occurs in early infancy, and data from clinical studies suggest that the inflammatory phenotype differs depending on the age at onset of disease.^{19,20} Until now, experimental studies elucidating the mechanisms underlying the pathogenesis of AD have been performed in adults. To address whether the age at which the first epicutaneous allergen sensitization takes place affects the nature of allergic skin inflammation, we exposed both neonatal (7-10 days old) and adult mice (6 weeks old) to HDM extract or vehicle control on the dorsal skin area for 3 consecutive weeks (Fig 1, A). Mice exposed to HDM in adulthood exhibited a significant increase in skin barrier function impairment (as measured by TEWL) (Fig 1, B) and disease severity score (Fig 1, C) compared with neonatal mice and vehicle-exposed adult controls. Neonatal epicutaneous allergen sensitization did not affect TEWL and no overt skin inflammation was observed. Histological analysis of the skin revealed an increase in epidermal thickness (acanthosis) in the skin of adult mice compared with neonates (Fig 1, D and E), indicating increased lichenification, which is a characteristic of AD.²¹ Systemic IgE and HDM-specific IgE responses were lower in neonatal mice (see Fig E1 in this article's Online Repository at www.jacionline.org), indicative of the early stages of B-cell development, which is in line with previous observations following neonatal allergen exposure in the lungs.²² Gene expression analysis revealed high levels of both IL-17a and TSLP transcripts in skin of adult but not neonatal mice (Fig 1, F).

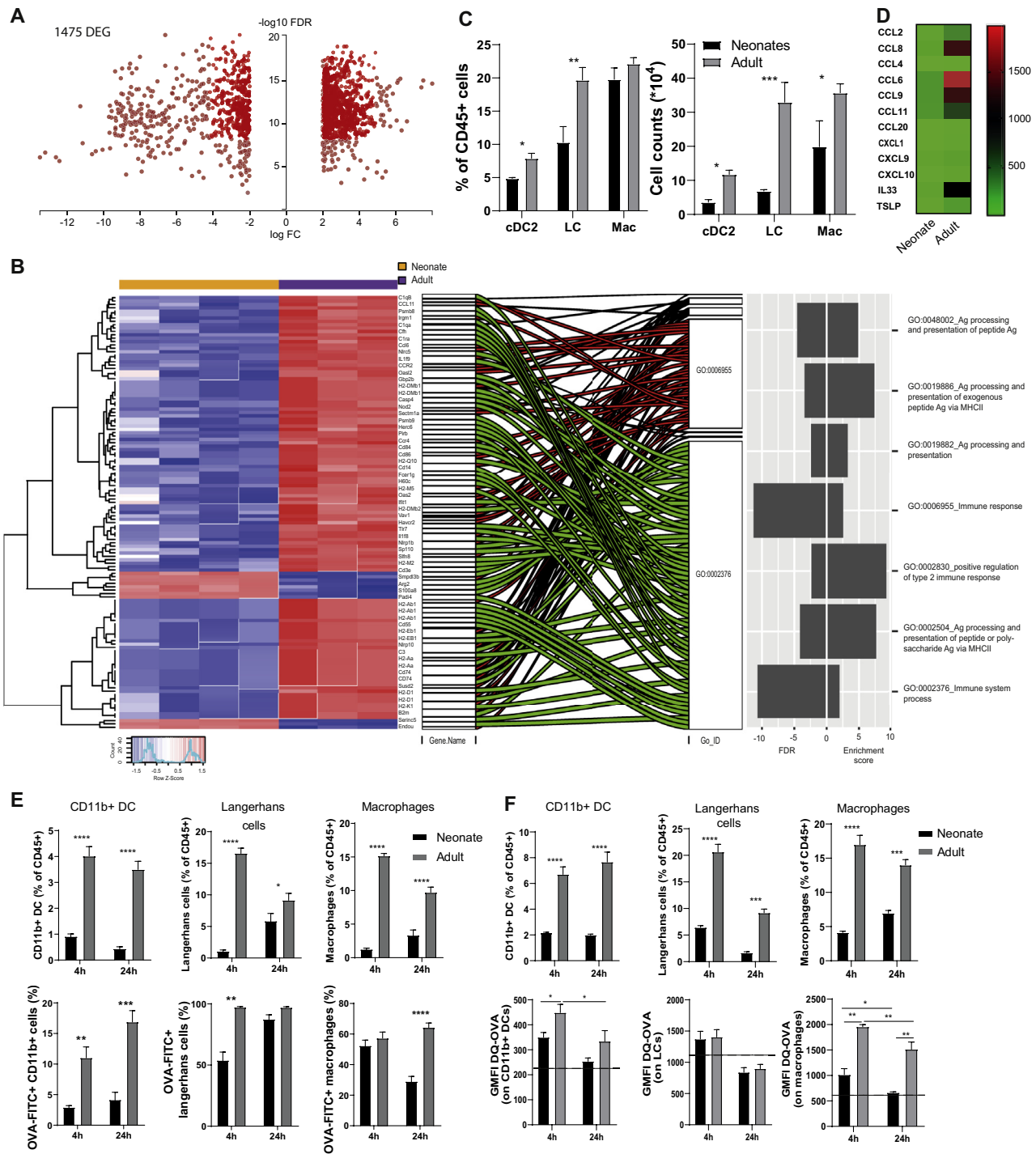


FIG 3. Age determines APC and chemokine seeding in the skin. **A**, Differentially expressed genes (DEGs) (false detection rate [FDR] < 0.05; log fold change = 2) after RNAseq of naive skin. **B**, Heatmap and alluvial plot of DEGs linked to select GO IDs enriched in adults. Mirrored box plot indicates $-\log_{10}$ FDR on left and enrichment score on right for each GO ID. **C**, APC frequency and absolute numbers in naive skin. **D**, Average count of chemokines and alarmins based on RNAseq. **E**, APC frequencies (of live) and frequency of OVA-FITC+ skin APCs (of indicated population at 4 hours and 24 hours postexposure). **F**, APC frequencies and mean fluorescence intensity of DQ-OVA in APCs (in indicated population) at 4 hours and 24 hours postexposure. Dashed lines indicate GMFI in fluorescence minus one controls. Results representative of data from 2 experiments. All data expressed as mean $\pm$ SEM ($n = 4$ for neonate and $n = 3$ for adult in Fig 3, **A**, **B**, and **D**; $n = 4$ neonates and $n = 7$ adults in Fig 3, **C**; $n = 6$ neonates and $n = 5$ adults in Fig 3, **E**; $n = 4$ for each group in Fig 3, **F**). Statistical significance determined by unpaired Student *t* test with Welch correction for Fig 3, **I**, **E**, and **F**, and 2-way ANOVA for Fig 3, **C**. GMFI, Geometric mean fluorescence intensity; LC, Langerhans cell; Mac, macrophage; TSLP, thymic stromal lymphopoietin. * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.

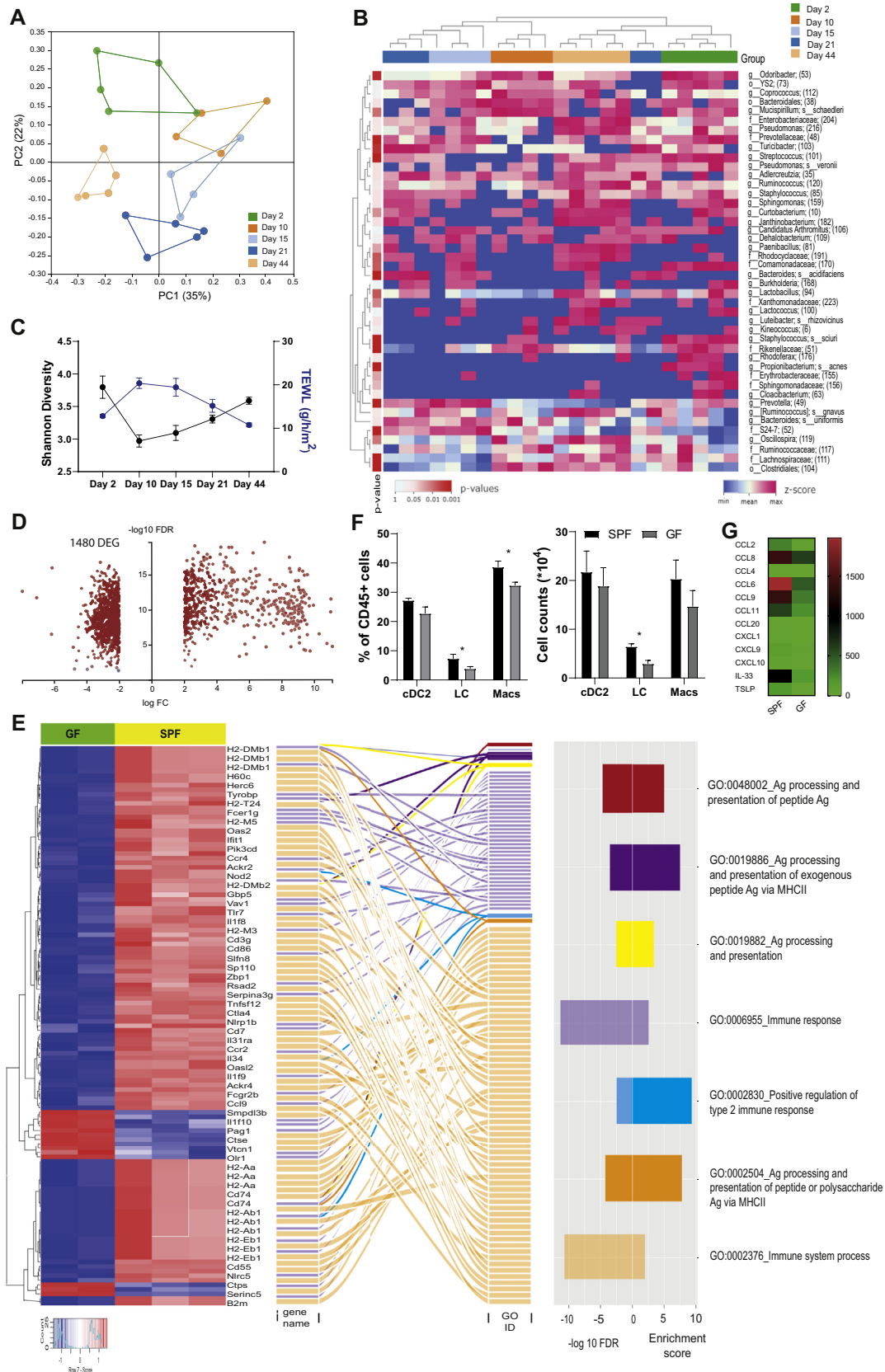


FIG 4. Development of the skin immunologic environment and barrier function is driven by microbial exposure. **A**, Principal-coordinates analysis plot based on Bray-Curtis distance of naive skin microbiome composition. **B**, Heatmap of genera abundances. Abundances are scaled on the basis of SD and centered

Restimulation of cells isolated from skin-draining lymph nodes (axillary and brachial) (Fig 1, *H*) and inguinal lymph nodes (Fig 1, *I*) led to secretion of IL-17a by effector cells from adult mice, but not neonates. However, both neonatal and adult T cells were equally capable of secreting IL-5. Comparatively, although IL-17a was not detected in the circulation of allergen-exposed adult mice, high levels were detected in neonatal serum (Fig 1, *G*), indicating that although neonates could mount an IL-17a response following allergen exposure, its localization was disorganized. Although no overt skin inflammation was observed in neonatal mice following epicutaneous allergen sensitization, analysis of skin inflammatory cell composition revealed increased eosinophil frequencies and absolute numbers in neonatal mice exposed to HDM extract (Fig 1, *J* and *K*). Moreover, adult mice exposed to HDM had increased total skin cell numbers and increased absolute eosinophil numbers compared with both vehicle-treated controls and sensitized neonatal mice (Fig 1, *J* and *K*). In contrast, cutaneous neutrophil frequencies and numbers were increased only in adult mice following HDM sensitization.

To ascertain whether the contrasting inflammatory responses in neonates and adults were unique to the skin, we next characterized the inflammatory response in the lung following inhaled allergen by mice epicutaneously sensitized as neonates or adults. Neonatally sensitized and adult-sensitized mice exhibited a robust airway eosinophilia; however, the airway inflammation in adult mice was dominated by neutrophils (Fig 1, *L*). In addition, this neutrophil-dominated airway response was accompanied by increased IL-17a protein levels in the bronchoalveolar lavage fluid (Fig 1, *M*). Some neutrophil recruitment to the airways was evident in neonates; however, they failed to produce IL-17a in the lungs and their capacity to induce an eosinophil-dominated allergic response in the lung was greater compared with adults, supporting the concept of a T_H2 -biased lung environment in early life. In addition, enhanced peribronchial inflammation was observed in lungs of neonatal mice (Fig 1, *N*).

Absence of overt skin inflammation is not due to lack of skin barrier impairment

Induction of oral tolerance occurs early in life and is dependent on the age of first antigen exposure.²³ Our data indicate that development of allergic skin inflammation is dependent on the age at first allergen exposure, raising the possibility that the absence of inflammation we observed was due to induction of tolerance. To investigate this, we sensitized neonatal mice with HDM extract or saline and then repeated this sensitization protocol with HDM extract in both groups when the mice had reached adulthood (Fig 2, *A*). Mice sensitized with HDM extract at both neonatal and adult age (HDM/HDM) exhibited an increased skin barrier function impairment, as measured by TEWL (Fig 2, *B*), and increased

disease severity score (Fig 2, *C*) compared with mice exposed to saline control at neonatal age and HDM extract at adult age (saline/HDM). Epidermal thickness (Fig 2, *D* and *E*) and cytokine secretion by effector cells in the skin-draining lymph nodes (Fig 2, *F*) were similar between the 2 groups. These results show that mice epicutaneously sensitized as neonates were able to develop allergic skin inflammation upon allergen exposure in adulthood, suggesting that the lack of overt skin inflammation in neonatal mice following epicutaneous allergen sensitization was most likely not due to the induction of tolerance to these allergens. Interestingly, these results highlight an exaggeration of the inflammatory response in the skin in mice sensitized in early life (Fig 2, *B* and *C*), suggestive of early-life immune priming but an inability to localize inflammation.

Genetic abnormalities in skin barrier function (eg, filaggrin mutations) and disruption of skin barrier by itching and scratching are common components of AD. To determine whether skin barrier disruption could induce allergic skin inflammation in neonatal mice, we performed tape stripping on neonatal mice before application of HDM extract or saline control (Fig 2, *G*). Tape stripping increased TEWL in both control and HDM-exposed mice, which is an indication of effective barrier disruption (Fig 2, *H*). However, no further increase was observed following the third and last sensitization round, and mice exposed to HDM extract did not show overt skin inflammation (Fig 2, *I*). Tape stripping combined with HDM sensitization increased epidermal thickness (Fig 2, *J* and *K*) and cytokine secretion by effector cells in the skin-draining lymph node compared with control (Fig 2, *L*). However, these responses are, although significantly different from controls, minor when compared with those observed in adult mice (Fig 1, *D*, *E*, and *H*). Tape stripping combined with HDM sensitization enhanced eosinophil and neutrophil numbers in the skin (Fig 2, *M* and *N*). These data suggest that tape stripping combined with epicutaneous allergen sensitization impairs skin barrier function, but this response does not persist and although cutaneous inflammatory cell numbers are enhanced, these responses do not lead to overt skin disease.

Age determines chemokine and APC seeding and function in the skin

To assess whether baseline differences in neonatal and adult skin immune composition could explain why neonatal mice do not develop allergic skin inflammation, we performed transcriptomic analysis on naive skin from neonatal (9-day-old) and adult (6-week-old) mice. Differential gene expression analysis revealed a total of 1475 genes that were differentially expressed (false detection rate < 0.05, log fold change > 2), of which 813 genes were upregulated and 662 genes were downregulated in adult compared with neonatal skin (Fig 3, *A*). GO enrichment

on mean per row. *P* values based on ANOVA analysis of groups. **C**, Microbial diversity based on Bray-Curtis distance analysis (black line) and TEWL (blue line). **D**, Differentially expressed genes (DEGs) (FDR < 0.05; log fold change = 2) after RNAseq of naive skin. **E**, Heatmap and alluvial plot of DEGs linked to select GO IDs enriched in adult SPF skin. Mirrored box plot indicates $-\log_{10}$ FDR on left and enrichment on right for each GO ID. **F**, APC frequency and absolute numbers in naive skin. **G**, Average count of chemokines and alarmins based on RNAseq data. Results representative of data from 2 different experiments. Data in Fig 4, *C* and *F*, expressed as mean $\pm$ SEM ($n = 5$ mice for day 2, day 21, and day 44 and $n = 4$ for day 10 and day 15 [Fig 4, *A-C*]; $n = 2$ for GF and $n = 3$ for SPF [Fig 4, *D* and *E*]; $n = 4$ for SPF and $n = 5$ for GF adults [Fig 4, *F* and *G*]). Statistical significance determined with 2-way ANOVA (Fig 4, *F*). *FDR*, False detection rate; *LC*, Langerhans cell; *Mac*s, macrophages; *PC*, principal coordinate; *TSLP*, thymic stromal lymphopoietin. * $P \leq .05$, ** $P \leq .01$.

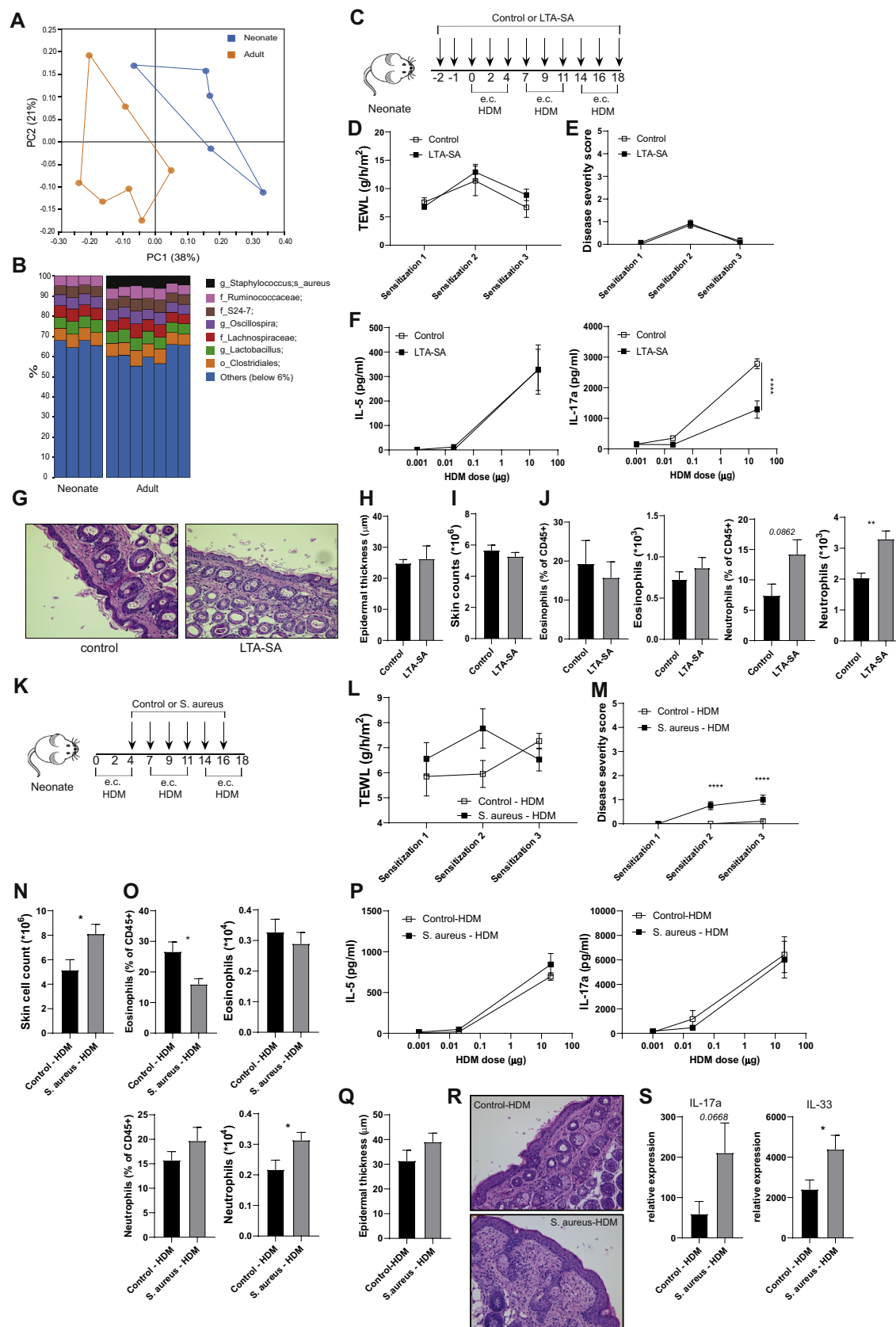


FIG 5. Development of skin inflammation is linked to *S aureus* presence in mice following allergen-induced AD. **A**, Principal-coordinates analysis plot (Bray-Curtis distance) of skin microbiome composition after sensitization. **B**, Relative abundance of OTUs. **C**, Experimental setup (arrow indicates LTA-SA exposure).

analysis revealed that genes belonging to the GO term “immune system process,” “immune response,” “positive regulation of type 2 immune response,” and several GO terms related to “antigen processing and presentation” were enriched in adult naive skin (Fig 3, B). These analyses provided a clear indication that one of the mechanisms underlying the impaired capacity of the neonatal skin to elicit inflammatory responses was due to an impaired antigen processing and presentation capacity. We thus investigated APCs in naive skin. In line with the transcriptomics data, we observed that the frequency and absolute numbers of CD11b⁺ DCs (cDC2), LCs, and macrophages were lower in naive skin from neonatal mice compared with adult mice (Fig 3, C; see Fig E2 and Fig E3, C, in this article’s Online Repository at www.jacionline.org). Cutaneous APCs in naive skin were found to gradually increase with age, with the largest increase between weanling (day 21) and adult age (Fig E3, A). Furthermore, we observed increased chemokine and alarmin expression in the skin of adult mice (Fig 3, D, and Fig E3, B), which are required for inflammatory cell recruitment and induction of an inflammatory response following HDM exposure. Analysis of the antigen uptake and processing capacity of skin APCs revealed a decrease in antigen uptake in CD11b⁺ DCs, LCs, and macrophages in neonatal mice (Fig 3, E; see Fig E3, E) and a lower antigen-processing capacity in CD11b⁺ DCs and macrophages (Fig 3, F; see Fig E3, F). These results demonstrate distinct differences in cutaneous immune cell seeding, APC function, and chemokine expression in naive neonatal skin, indicating that these factors are imperative for the development of allergic skin inflammation.

Microbial exposure drives development of the immunologic environment in the skin

Microbial exposure in early life occurs concomitantly with and has an impact on the immune system development. We set out to assess whether the skin microbiome is important for the development of the immunologic environment in the skin. Principal-coordinate analysis of longitudinal sampling of the naive murine skin microbiome from birth to adulthood indicated that the skin microbiome composition of different mice at a given time point after birth is more similar than the skin microbiome of one animal at different ages (Fig 4, A). In addition, hierarchical clustering of samples based on relative genera abundances suggests that the skin microbiome at day 2 has similarities with that of adult mice (Fig 4, B). When assessing skin microbiome diversity and skin barrier function, we observed that bacterial diversity was inversely associated with impaired skin barrier function (Fig 4, C). Moreover, *prevotellaceae*, *streptococcus*, *lactobacillus*, and *propionibacterium* (among others) were lost during

the dip in diversity. Interestingly, only *lactobacillus* was regained over the course of development (see Table E1 in this article’s Online Repository at www.jacionline.org). Furthermore, depletion of the skin microbiome in neonatal mice during epicutaneous allergen sensitization using topical application of broad-spectrum antibiotics increased TEWL levels, indicative of impaired barrier function, but did not affect skin disease (see Fig E4, A-C, in this article’s Online Repository at www.jacionline.org). Topical application of antibiotics during HDM sensitization did not affect cytokine secretion by effector cells in the skin-draining lymph node, epidermal thickness, or cutaneous gene expression compared with vehicle control (Fig E4, D-F). Interestingly, skin barrier function was similar in naive neonatal specific pathogen-free (SPF) and GF mice, whereas absence of the microbiome led to impaired skin barrier function as the mice aged (Fig E4, G). Together, these results suggest that skin microbiome diversity is important for establishment of the skin barrier.

To further assess the importance of the microbiome in the development of the immunologic environment in the skin, we performed transcriptomic analysis on naive skin from adult SPF and GF mice. Differential gene expression analysis revealed 1480 differentially expressed genes (false detection rate < 0.05, log fold change > 2), of which 614 genes were upregulated and 866 genes were downregulated in GF compared with SPF mouse skin (Fig 4, D). GO terms enriched in adult naive SPF skin in comparison to adult GF skin (Fig 4, E) were similar to the enrichment observed between adult and neonatal SPF mice (Fig 3, B). In line with these results, LC and macrophage frequencies were decreased in skin from naive adult GF mice (Fig 4, F; see Fig E3, D) along with the expression of chemokines and alarmins (Fig 4, G; see Fig E3, B). Cohousing of naive weanling (day 21) GF mice with age- and sex-matched naive SPF mice, and thereby their acquisition of an SPF-like microbiome, led to similar, or even enhanced, levels of cutaneous APCs and chemokines/alarmins in cohoused GF mice (see Fig E5, A-D, in this article’s Online Repository at www.jacionline.org). These results provide evidence that the skin microbiome is important not only for the development of the immunologic environment in the skin but also for skin barrier function.

S aureus can induce overt skin inflammation in neonatal mice

Our results demonstrate that postnatally the microbiome drives chemokine production and seeding of APCs in the skin. Allergic skin inflammation is often characterized by changes in the microbial composition of the skin, and patients with AD are frequently colonized with *S aureus*.^{24,25} We determined whether epicutaneous allergen sensitization alters the skin microbiome

D, TEWL. E, Disease severity score. F, IL-5 and IL-17a cytokine levels in supernatant of HDM-stimulated skin-draining lymph node cells. G, Representative H&E-stained skin sections; 100× magnification. H, Quantification of epidermal thickness. I, Total skin cell numbers. J, Eosinophil and neutrophil numbers. K, Experimental setup (arrow indicates *S aureus* exposure). L, TEWL. M, Disease severity score. N, Total skin cell numbers. O, Eosinophil and neutrophil numbers. P, IL-5 and IL-17a cytokine levels in supernatant of HDM-stimulated skin-draining lymph node cells. Q, Quantification of epidermal thickness. R, Representative H&E-stained skin sections; 100× magnification. S, Relative IL-17a and IL-33 mRNA expression levels in skin. Results representative of data from 2 different experiments. All data expressed as mean ± SEM (n = 5 neonates [Fig 5, A and B]; n = 7 mice control and n = 10 for LTA-SA treatment [Fig 5, C-J]; n = 5 mice for each group [Fig 5, K-R]). Statistical significance determined with unpaired Student *t* test with Welch correction. In Fig 5 (D-F and L, M, P), 2-way ANOVA test was used. *e.c.*, Epicutaneous; *PC*, principal coordinate; **P* ≤ .05, ***P* ≤ .01, and ****P* ≤ .001 compared with control condition.

in neonatal and adult mice. Principal-coordinate analysis revealed that the skin microbiome of neonatal mice following allergen sensitization is different to that of adult mice (Fig 5, A). We observed *S aureus* presence on the inflamed skin of adult mice (Fig 5, B), whereas it was below the limit of detection on the skin of neonatal mice. No differences in richness or diversity were observed between neonatal and adult mouse skin microbiome (data not shown). These data indicate that skin inflammation creates a habitat that is suited to colonization by *S aureus*.

We next determined whether TLR2-mediated immune stimulation with lipoteichoic acid from *S aureus* (LTA-SA), a major immunostimulatory component of gram-positive bacteria, could induce allergic skin inflammation in neonatal mice (Fig 5, C). Treatment of neonatal mice with LTA-SA during epicutaneous allergen sensitization did not alter skin barrier function, disease severity score, or epidermal thickness (Fig 5, D, E, G, and H). However, IL-17a cytokine secretion by effector cells in the skin-draining lymph node was decreased following LTA-SA exposure (Fig 5, F). Analysis of inflammatory skin cell populations revealed an increase in neutrophils, whereas eosinophil levels remained the same (Fig 5, I and J). These data suggest that substantial TLR2 stimulation can induce innate immune responsiveness in the skin of neonatal mice; however, overt allergic skin disease does not develop.

To assess the influence of live *S aureus* exposure on the development of allergic skin inflammation, we exposed neonatal mice to *S aureus* after the first week of epicutaneous HDM sensitization (Fig 5, K). Skin disease severity was enhanced by *S aureus* colonization, whereas skin barrier function was not significantly impaired (Fig 5, L and M). Moreover, analysis of cutaneous immune cell composition revealed a significant decrease in eosinophil frequencies and an increase in neutrophil numbers (Fig 5, N and O). *S aureus* exposure did not affect systemic immune responses or epidermal thickness (Fig 5, P-R). However, IL-17a and IL-33 gene expressions were increased in the skin of mice exposed to *S aureus*, which is in line with the observed increase in neutrophils (Fig 5, S). Taken together, these data indicate that topical *S aureus* exposure in neonatal mice during allergen sensitization contributes to the development of overt skin inflammation and an enhanced neutrophilic inflammatory response. A similar response was observed in adult mice exposed to *S aureus*, with no differences in epidermal barrier function, but an increase in disease severity (see Fig E6, A-C, in this article's Online Repository at www.jacionline.org).

DISCUSSION

Our study shows that although adult mice can develop a profound allergic skin response following epicutaneous exposure to HDM, neonatal mice do not. This inflammatory response developed in adults, even with intact skin, suggesting that mechanical skin barrier impairment is not a prerequisite to induce AD. In line with these results, it has been previously demonstrated that allergens with protease activity, such as HDM (Der p 1), can induce AD without prior mechanical skin barrier disruption.²⁶ Mechanical barrier disruption before allergen sensitization has been shown to enhance the allergic inflammatory response in the skin of mice.²⁶ It is suggested that both the enhanced permeability of the skin, allowing for more allergen penetration, and the increased inflammatory response, leading to subsequent recruitment and polarization of immune cells,

induced by the mechanical barrier disruption, exaggerate inflammation.²⁷⁻²⁹ Although we disrupted the skin barrier by tape stripping, neonatal mice still did not develop overt signs of allergic skin disease following allergen exposure. Histological examination did reveal an increase in epidermal thickening, which could be an indication of enhanced epidermal keratinocyte proliferation in response to a combination of tape stripping and HDM exposure. Moreover, cutaneous eosinophil and neutrophil numbers were found to be increased following tape stripping and HDM exposure. Because of ethical constraints concerning the sampling of healthy neonatal skin, there are very limited data available on the presence of APCs in the skin of healthy human neonates. Very early onset (between age 3 months and 2 years) of AD is seen in 60% to 80% of the cases. However, in less than half the patients with very early onset AD, the disease is continuous, whereas the other part of the children in this group goes into complete remission before age 2 years. The short-term mild inflammatory response that we observed in neonatal mice, following mechanical barrier disruption and exposure to LTA-SA, might share similarities with "eczema" that intermittently presents in young infants, but does not develop into continuous disease.

To mount an inflammatory response it is imperative that APCs are present to take up, process, and present antigens. We report that the skin of naive neonatal mice contains lower numbers of APCs and chemokines compared with adult mice. Moreover, these APCs exhibit a decreased antigen uptake and processing capacity. This lack of APC seeding and function in neonatal mouse skin underlies the impaired inflammatory response following allergen exposure. DC recruitment and migration are coordinated by chemokines.³⁰ Nagao et al³¹ demonstrated that chemokine production by hair follicle keratinocytes regulates the trafficking of DCs in the skin. Hair follicles provide a skin-specific niche for immune cells such as monocytes and monocyte-derived LC precursors.^{31,32} In addition, hair follicles harbor various T lymphocytes, such as skin-resident Trm cells, which are responsible for long-term skin immunity,³³ and regulatory T (Treg) cells, which accumulate in the hair follicle and reside near the hair bulge.^{34,35} Tordesillas et al³⁶ recently demonstrated that antigen uptake following topical allergen application was absent in hairless mice. Together with the fact that hair follicles exhibit a facilitating role in both DC and T-cell trafficking, this suggests that there is an important role for the hair follicle in antigen acquisition and presentation under certain conditions.^{31,37} Whether incomplete hair follicle development in the first 2 weeks of life³⁸ is involved in the decreased capacity of neonatal APCs to acquire and process antigen remains to be delineated.

The microbiota plays a fundamental role in the development of homeostatic immunity in barrier tissues such as the lung and gut.^{3,39} The formation of the skin microbiota starts during birth and continues to develop over the first weeks and months.^{40,41} Cutaneous bacteria are distributed throughout the epidermal layers and in the hair follicles, of which the latter is the preferred colonization site for commensals.^{42,43} Chemokine interactions (eg, Ccl20-Ccr6) have been shown to link hair follicle development and local colonization with commensal microbes to Treg-cell migration in neonatal skin.³⁵ In turn, this influx of Treg cells mediates tolerance to skin commensal microbes in early life.⁴⁴ Our study provides evidence that the chemokine profile and APC populations are in naive skin from neonatal SPF mice and

adult GF mice, compared with skin from adult SPF mice. Cohousing of GF mice with SPF mice rescued chemokine expression and seeding of APCs in the skin, suggesting that microbial colonization is central to these processes. Treg-cell numbers are lower in the skin of 13-day-old GF mice compared with their SPF controls.³⁵ Interestingly, the Treg-cell numbers in neonatal GF mice approached the Treg-cell numbers in skin of 6-day-old SPF mice in a study by Scharschmidt et al,³⁵ suggesting that indeed the microbiome plays an important role in immune cell seeding of the skin in early life.

The skin microbiome is an important player in the pathogenesis of AD.^{45,46} *S aureus* is a dominant member of the cutaneous bacterial composition in most patients with AD and has been linked to severity of disease.^{24,25,46} Analysis of the adult mouse skin microbiome following epicutaneous allergen sensitization revealed the presence of *S aureus*, a known pathobiont, which can bloom in an inflammatory environment. *S aureus* was not sufficiently abundant to be detected in neonatal mice, which lacked skin inflammation following allergen sensitization. Exposure of primary keratinocytes *in vitro* to LTA-SA was shown to induce essential components of the innate immune response, such as cytokine and chemokine expression, which in turn could contribute to immune cell infiltration.⁴⁷ We observed that topical exposure of neonatal skin to LTA-SA *in vivo* during allergen sensitization increased neutrophil numbers in the skin, which is similar to observations made by Brauweiler et al⁴⁷ in adult murine skin. Although LTA-SA exposure alone did not induce overt skin disease, topical exposure to live *S aureus* combined with HDM sensitization initiated a skin rash. Cutaneous eosinophil frequencies were decreased, whereas neutrophil numbers and cutaneous IL-17a gene expression were increased, suggesting a potential switch from an eosinophil-dominated cutaneous inflammation to a more neutrophilic inflammatory response.

AD in early life can co-occur with other atopic diseases such as food allergies and allergic asthma. Our study demonstrates that although epicutaneous allergen sensitization does not induce overt skin inflammation in neonates, these mice are able to elicit an allergic inflammatory response in the airways following intranasal allergen challenge. We provide evidence that in the naive state, skin barrier function in neonatal mice is impaired in the first 2 weeks of life, which could lead to enhanced penetration of allergen without induction of skin disease. In line with these results, Kelleher et al⁴⁸ demonstrated that children with an impaired skin barrier function at birth, regardless of the presence of AD, were at an increased risk of developing food allergies by age 2 years. However, the mechanisms underlying this observation and the potential implications for the development of allergic asthma following impaired skin barrier function in early life remain to be determined.

The nature and severity of the allergic inflammatory response in the lung following skin allergen sensitization in neonatal compared with adult mice differ. Adult mice exhibit a mixed T_H2/T_H17 inflammatory profile in the skin and the lung, which is also apparent in the effector cell responses in the lymph nodes. Epicutaneous allergen sensitization in adult mice using HDM, ovalbumin, or papain was shown to induce both T_H2 and T_H17 responses,⁴⁹⁻⁵¹ which is in line with our findings. Interestingly, serum IL-17a levels were undetectable in our adult mice, whereas we detected high circulating IL-17a levels in neonates following allergen sensitization. The source and implications of the serum IL-17a in the neonates remain to be determined, but shows that

these mice are competent to induce an IL-17a response, but due to immature and disorganized immune development, this IL-17a production does not occur at the site of allergen exposure, that is, the skin.

Our data provide insights into the early immune signals required to elicit allergic responses in early life. Importantly, distinct immunologic rules govern the skin and lung in early life and adulthood. We demonstrate that age-related microbial-driven chemokine production and seeding of the skin with APCs is central to the development of allergic skin inflammation.

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Key messages:

- Neonatal skin exhibits impaired chemokine and alarmin production and low numbers of skin-resident APCs.
- Formation of skin barrier function and immune maturation is driven by the skin microbiome.

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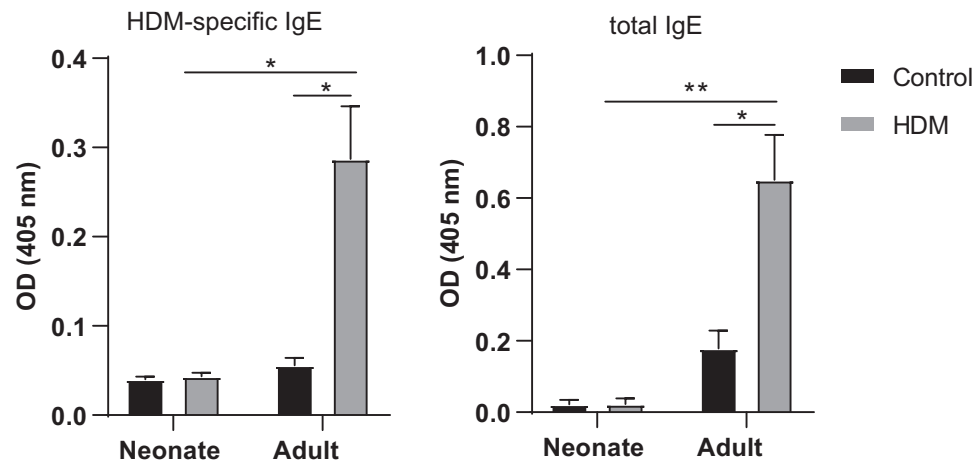


FIG E1. Age of first epicutaneous allergen sensitization determines serum antibody levels and lung inflammation. Serum IgE and HDM-specific IgE antibody levels were determined at the end of the third sensitization round. Data are expressed as mean $\pm$ SEM ($n = 5$ mice for each group). Statistical significance was determined with unpaired Student t test with Welch correction. * $P \leq .05$, ** $P \leq .01$.

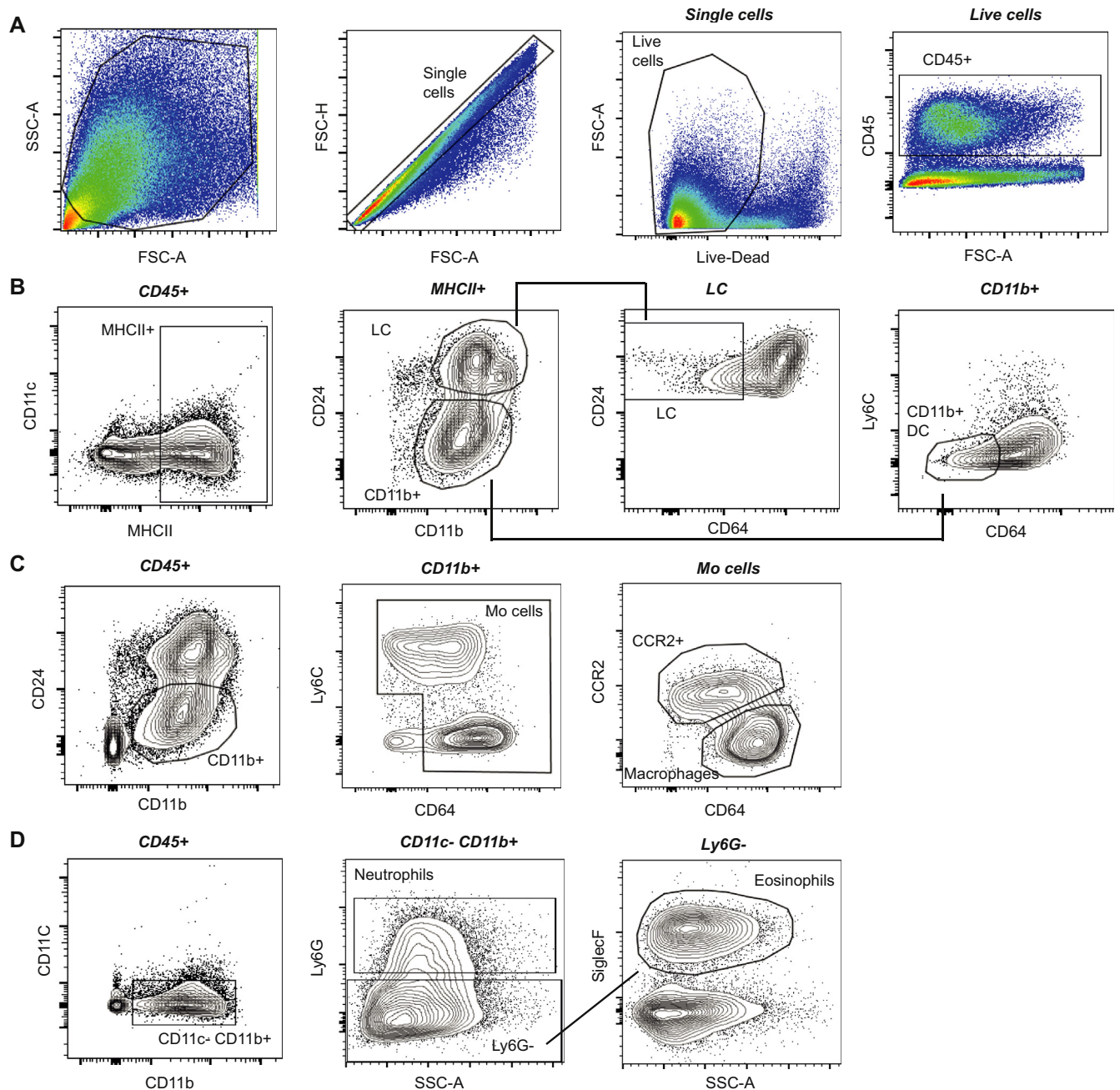


FIG E2. Gating strategy for skin cell composition analysis. **A**, Pregating strategy for viable, CD45⁺ single cells in the skin. **B**, LCs are gated as CD45⁺MHCII⁺CD24⁺CD11b⁺CD64⁻ cells; CD11b⁺ DCs are gated as CD45⁺MHCII⁺CD24⁻CD11b⁺CD64⁺Ly6C⁻ cells. **C**, Macrophages are gated as CD45⁺CD24⁻CD11b⁺CD64⁺CCR2⁺ cells. **D**, Neutrophils are gated as CD45⁺CD11c⁻CD11b⁺Ly6G⁺ cells; eosinophils are gated as CD45⁺CD11c⁻CD11b⁺Ly6G⁻SiglecF⁺ cells. *FSC-A*, forward scatter-area; *FSC-H*, Forward scatter-height; *SSC-A*, side scatter-area.

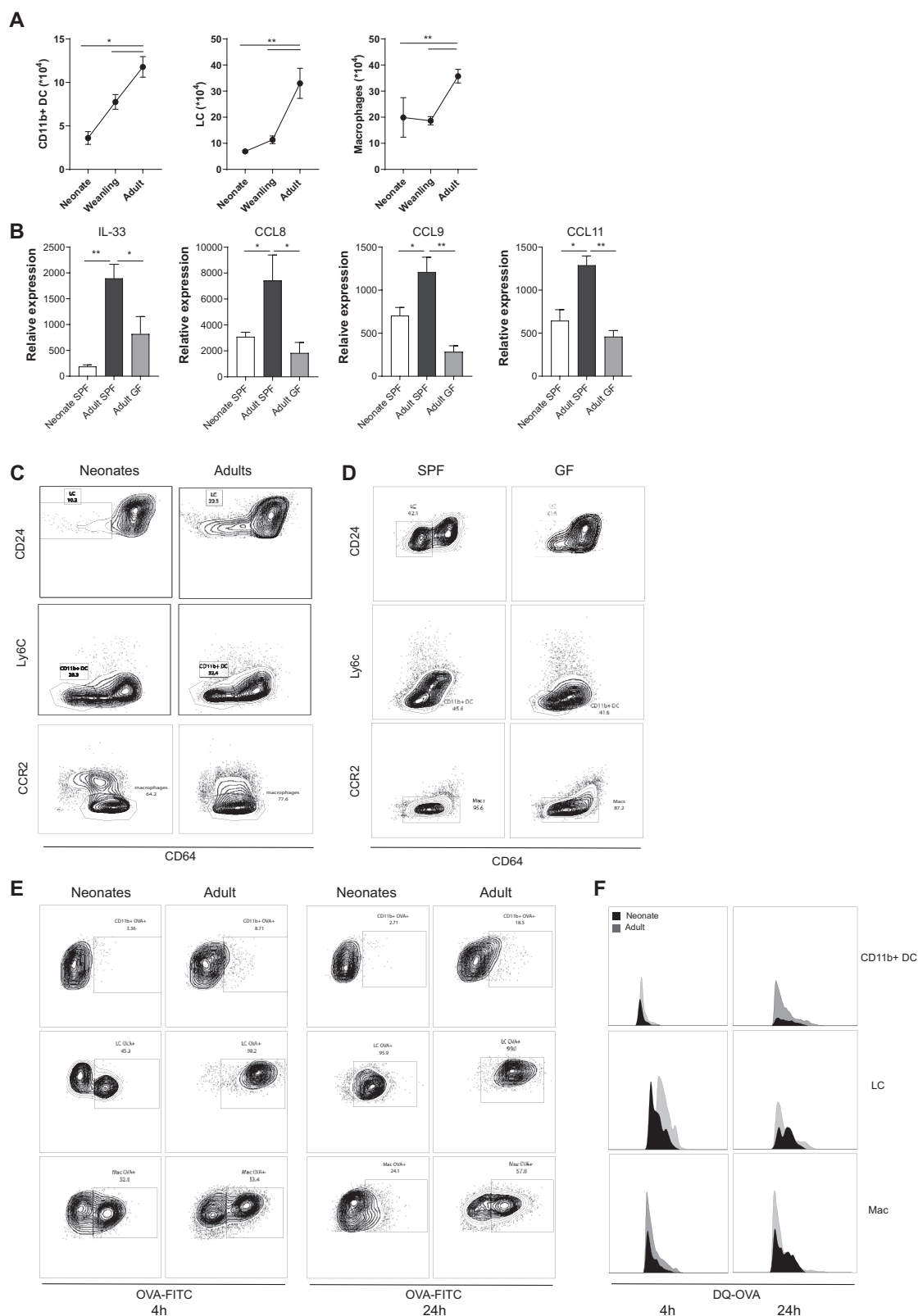


FIG E3. Chemokine expression and APC frequencies in naive skin. **A**, APC frequencies in naive skin of neonatal (day 9), weanling (day 21), and adult (day 44) SPF mice. **B**, Validation of mRNA expression levels of IL-33, CCL8, CCL9, and CCL11 in naive skin of neonatal and adult SPF mice and adult GF mice. **C** and **D**, Representative flow plots of (Fig E3, C) neonatal vs adult SPF or (Fig E3, D) adult SPF vs GF skin APCs. **E**, Representative flow plots of OVA-FITC+ APCs. **F**, Representative histograms of DQ-OVA signal intensity in neonatal vs adult APC. Results are representative of data generated in 2 different experiments. Data are expressed as mean $\pm$ SEM ($n = 6$ mice for each group). Statistical significance was determined with 2-way ANOVA test (Fig E3, A) or unpaired Student t test (Fig E3, B). Mac, Macrophage. * $P \leq .05$, ** $P \leq .01$.

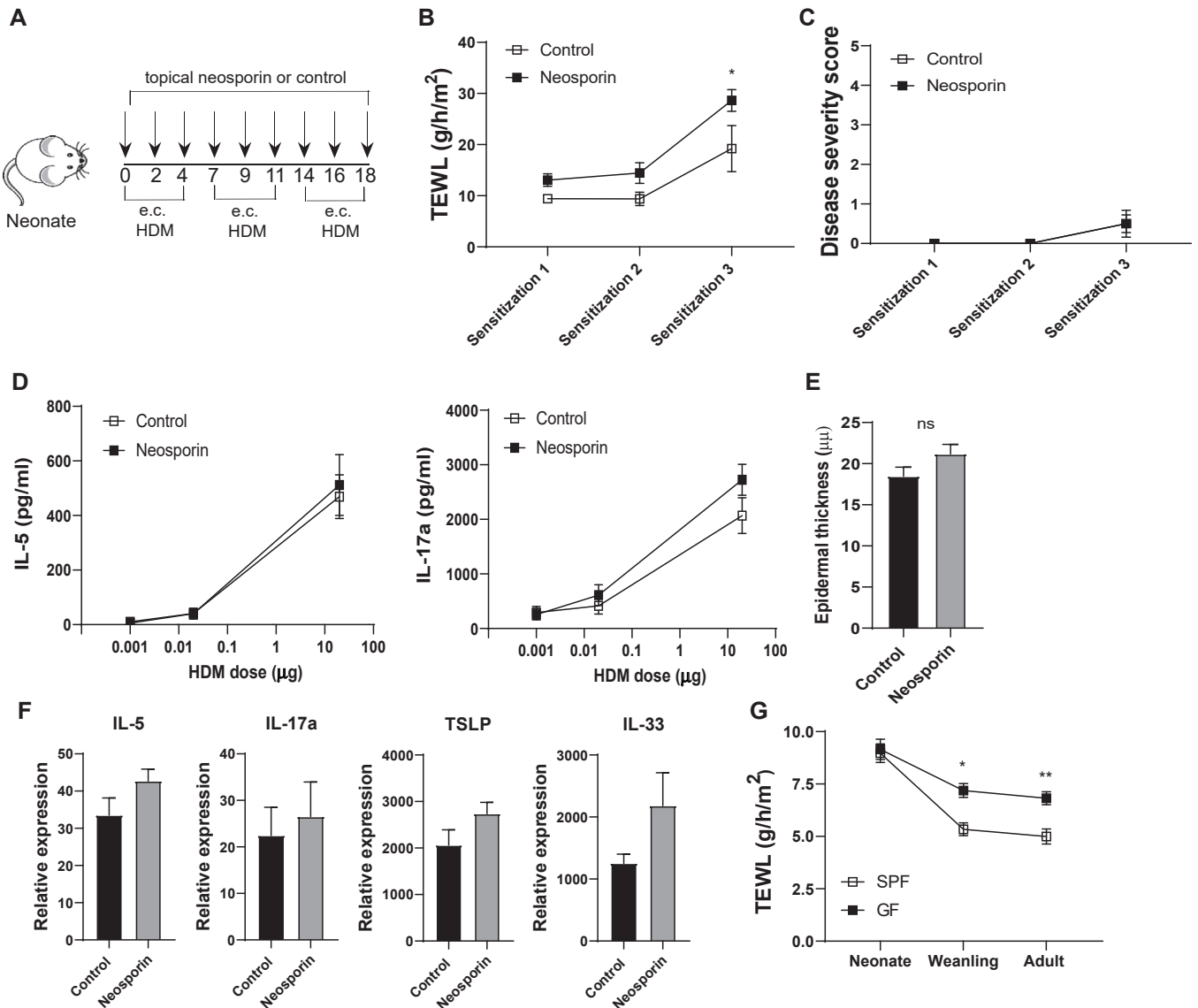


FIG E4. Neonatal skin microbiome depletion impairs skin barrier function. **A**, Experimental model. Neonatal mice were topically treated with Neosporin or control 3 times per week before application of HDM epicutaneously. **(B)** TEWL and **(C)** skin disease severity score at the end of each sensitization round. **D**, IL-5 and IL-17a cytokine levels in supernatant of HDM-stimulated cells isolated from skin-draining lymph nodes (brachial and axillary) at the end of the last sensitization round. **E**, Quantification of epidermal thickness on H&E-stained skin tissue sections. **F**, mRNA expression levels of IL-5, IL-17a, TSLP, and IL-33 in the skin. **G**, TEWL measurement on naive skin of neonatal (day 9), weanling (day 21), and adult (day 44) SPF and GF mice. Results are representative of data generated in 2 different experiments. Data are expressed as mean $\pm$ SEM ($n = 6$ mice for each group [Fig E4, B-F] and $n = 10$ mice for each group in Fig E4, G). e.c., Epicutaneous; ns, not significant; TSLP, thymic stromal lymphopoietin. Statistical significance was determined with 2-way ANOVA test (Fig E4, B-D, G) or with unpaired Student t test (Fig E4, E and F). * $P \leq .05$, ** $P \leq .01$.

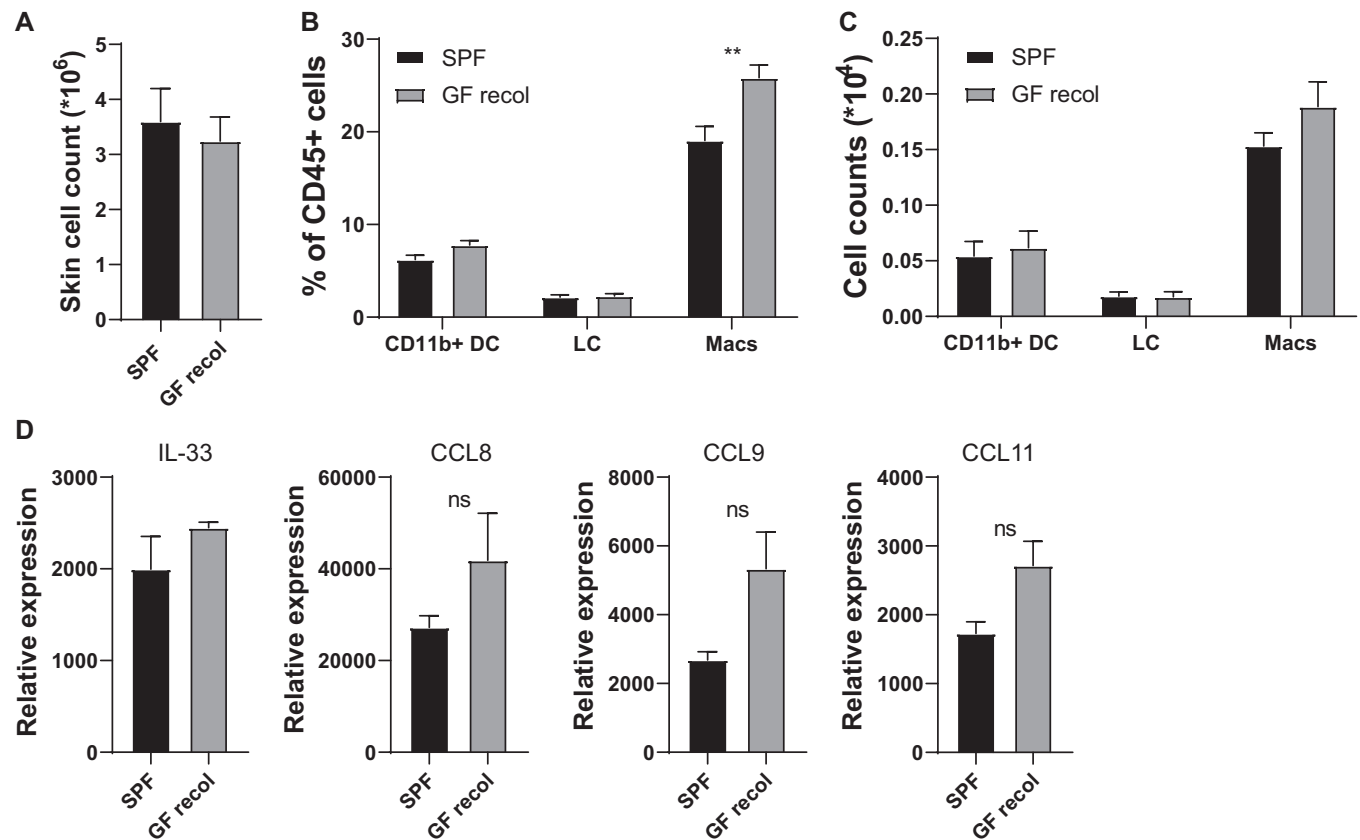


FIG E5. Cohousing of GF mice with same-age SPF mice rescues APC and chemokine seeding in the skin. **A**, Total cell counts in skin of 42-day-old SPF mice and cohoused GF mice. **B**, APC frequencies (% of CD45⁺ cells) and **C**, absolute cell numbers in naive skin of SPF mice and cohoused GF mice. **D**, mRNA expression levels of IL-33, CCL8, CCL9, and CCL11 in naive skin of SPF mice and cohoused GF mice. Results are representative of data generated in 2 different experiments. Data are expressed as mean $\pm$ SEM ($n = 5$ mice for each group). Statistical significance was determined with unpaired Student t test. LC, Langerhans cell; Macs, macrophage; ns, not significant; recol, recolonization. ** $P \leq .01$.

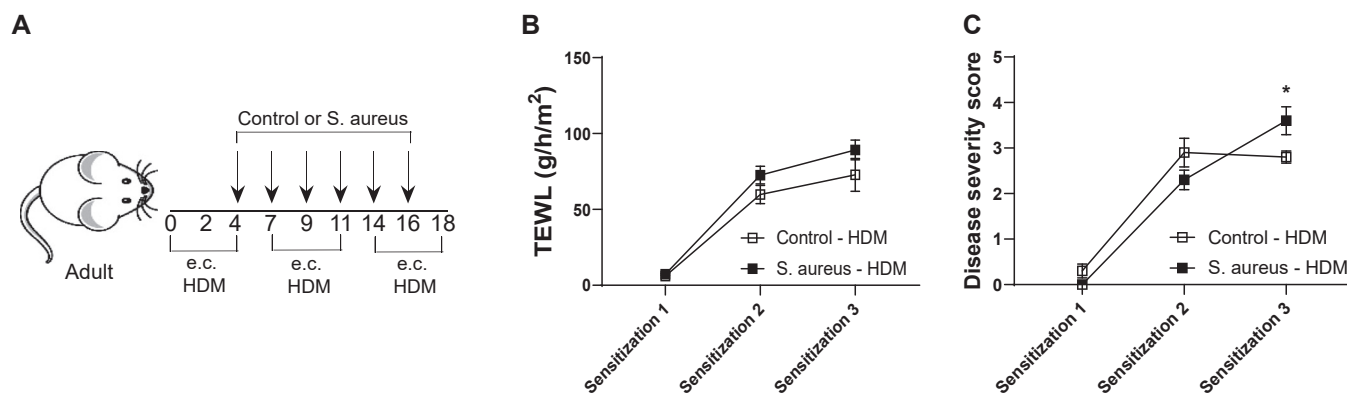


FIG E6. Topical *S aureus* in adult mice enhances allergic skin disease severity. **A**, Experimental setup. Adult mice are topically treated with live *S aureus* or control (at each arrow) throughout the epicutaneous allergen sensitization rounds. **(B)** TEWL and **(C)** skin disease severity score at the end of each sensitization round. Data are expressed as mean $\pm$ SEM ($n = 10$ mice for each group). Statistical significance was determined with 2-way ANOVA test. *e.c.*, Epicutaneous. * $P \leq .05$ compared with control condition.

TABLE E1. Differential analysis skin microbiome

OTU	Abundance	Log2 fold change	P value	Adjusted P value
<i>Day 2 vs day 10 (decreased)</i>				
g_Propionibacterium	37.41	−4.09	8.31×10^{-12}	2.66×10^{-10}
g_Staphylococcus	603.57	−5.76	3.69×10^{-11}	7.60×10^{-10}
g_Streptococcus	131.03	−3.65	2.83×10^{-10}	4.53×10^{-09}
g_Flavobacterium	126.09	−4.61	4.26×10^{-09}	5.85×10^{-08}
g_Lactobacillus	602.02	−2.48	1.33×10^{-07}	1.60×10^{-06}
g_Corynebacterium	55.02	−4.11	1.09×10^{-05}	1.04×10^{-04}
f_Prevotellaceae	51.71	−2.26	1.18×10^{-04}	8.06×10^{-04}
g_Bacillus	17.25	−3.71	1.02×10^{-03}	5.05×10^{-03}
g_Enterococcus	16.69	−5.08	3.86×10^{-03}	1.37×10^{-02}
<i>Day 21 vs day 44 (increased)</i>				
g_Bacillus	18.57	2.30	2.59×10^{-07}	4.68×10^{-06}
g_Lactobacillus	473.81	1.047	3.89×10^{-06}	4.95×10^{-05}