Materials and Methods

Animals

Female SKH1 mice (6-7 weeks old, Charles River) were purchased and allowed to acclimate for at least one week. SKH1 mice are the most commonly used hairless mouse strain and have previously been used to assess the skin microbiome (SanMiguel *et al.*, 2017). Mice were randomly assigned to an exposure group and identified with tail markings made with a permanent marker. Mice were housed (3-5/cage) in ventilated plastic shoebox cages with autoclaved bedding and crinkle nest material. Harlan NIH-31 modified 6% irradiated rodent diet and tap water were available *ad libitum*. Housing facilities were maintained with a 12-hour light/dark cycle. Specific measures were taken to control microorganisms: a dedicated bioexclusion room with limited access was used for the study, all equipment was wiped down with 70% ethanol prior to use, and animal cages were changed twice per week. All procedures were conducted under a class II type B2 biological safety cabinet (Baker SterilchemGARD). All animal experiments were performed in the AAALAC International accredited National Institute for Occupational Safety and Health (NIOSH) animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

Triclosan Exposures

Triclosan (CAS# 3380-34-5) was purchased from Calbiochem (EMD Millipore Corp.). Acetone (CAS# 67-41-1) was purchased from Sigma-Aldrich. Mice (5-6/group) were exposed once per day for 7 consecutive days to acetone (vehicle control) or to triclosan (0.5%, 2%) dissolved in acetone (w/v) on the dorsal back skin (100 µL/mouse). An additional unexposed (naïve) control group was included for the microbiome experiments. The concentrations were selected based on previous

study findings and determined to be non-toxic (Anderson *et al.*, 2013). Acetone was selected as the vehicle based on solubility and previous uses in evaluations of chemical exposures.

TEWL Measurements

Trans-epidermal water loss (TEWL) was determined daily prior to exposure (0%, 0.5%, 2%) using a VapoMeter (Delfin) and small adapter on mouse dorsal skin per manufacturer's instructions.

Euthanasia and Skin Collection

Animals were euthanized by CO_2 inhalation 24 hours after the final exposure. Back skin (1 cm^2) was fixed in 4% paraformaldehyde or 10% formalin for immunofluorescence or histology, respectively. Back skin (1 cm^2) was collected for western blot into tubes containing a steel bead and 750 µL T-PER with Halt Protease and Phosphatase Inhibitor cocktail and 0.5 M EDTA. Samples were processed on a TissueLyser II and supernatant was collected and frozen at -80 °C until analyzed. Back skin (1 cm^2) was collected for gene expression into tubes containing 500 µL RNAlater (Invitrogen) and frozen at -80 °C until processed.

Histology

Formalin-fixed paraffin-embedded mouse skin (5 µm) was mounted on slides and stained with hematoxylin and eosin following standard procedures (1 slide/animal). Slides were brightfield imaged on an Olympus VS120 slide scanner at 40X. Epidermal thickness was measured using ImageJ from 1 random view/slide and 3 measurements per view were taken and averaged. For histopathology, prepared slides were transferred to Vet Path Services, Inc. Provantis™ pathology software v10.1.0.1 was utilized for data capture. Stained histologic sections were examined by light microscopy and observations were entered into Provantis™ by the non-blinded Study Pathologist. Histologic sections were of adequate size and quality for detailed evaluation. Histopathology grades were assigned as grade 1 (minimal), grade 2 (mild), grade 3 (moderate),

grade 4 (marked), or grade 5 (severe) based on an increasing extent of change, unless otherwise specified.

Immunofluorescence Imaging

Fixed mouse skin was cryopreserved in 30% sucrose, frozen in optimal cutting temperature compound, cryosectioned (5 µm), and mounted on slides. Skin sections (1 section/animal for each protein of interest) were washed with PBS, blocked with blocking buffer (3% bovine serum albumin with 0.1% Triton in PBS) at room temperature for 1 hour, and stained with primary antibody diluted in blocking buffer at 4 °C overnight. The following primary antibodies were used: FLG (1:200; BioLegend 905804), FLG2 (1:200; NBP1-91901), keratin 10 (KRT10) (1:400; ab76318), and keratin 14 (KRT14) (1:500; NBP2-67585). Slides were washed, stained with secondary antibody (Alexa Fluor 594, 1:300) at room temperature for 1 hour, washed, and stained with DAPI Fluoromount-G. Images were acquired at 20X using an Olympus VS120 Slide Scanner. Negative controls with secondary antibody only were included for each sample and consistently showed low background fluorescence (Supplemental Figure 1). Distribution (µm) was measured using ImageJ from 1 random view/slide and 3 measurements per view were taken and averaged.

Protein Analysis

Total protein was quantified using the BCA protein assay (Pierce) per manufacturer's instructions. Select proteins of interest were quantified using a capillary immunoassay. Optimal sample and antibody concentrations were determined using a linear range finding assay. Samples were prepared in sample buffer and fluorescent master mix, then denatured at 95 °C for 5 minutes. Samples, antibodies (FLG, 0.4 mg/mL; FLG2, 0.4 mg/mL; KRT10, 0.2 mg/mL; KRT14, 0.4 mg/mL), and reagents were loaded and run on a Wes machine (ProteinSimple) using total protein 12-230 kDa assay with 18 sec. stacking matrix load time and 30 min. separation time. The

ProteinSimple Anti-Rabbit and Total Protein Detection Modules were used. Peaks were automatically detected and manually inspected to confirm a peak signal-to-noise (S/N) ratio above 10 and peak height/baseline ratio above 3. Area under the curve was calculated for total protein and protein of interest using Compass software (ProteinSimple). Protein of interest was normalized to total protein. Proteins evaluated include FLG (1:50; BioLegend 905804), FLG2 (1:50; Bethyl A305-861A-M), KRT10 (1:150; ab76318), and KRT14 (1:50; ab197893).

Gene Expression Analysis

Total RNA was isolated from skin using the RNeasy kit per manufacturer's instructions (Qiagen). A QIAcube (Qiagen) automated RNA isolation machine was utilized in conjunction with the RNA isolation kit. The concentration and purity of the RNA were determined using a NanoDrop Spectrophotometer (Thermo Scientific). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per manufacturer's instructions. TaqMan Fast Universal PCR Master Mix (Applied Biosystems), cDNA, and gene-specific primers (TaqMan Gene Expression Assays) were combined and real-time quantitative PCR (qPCR) was performed per manufacturer's instructions. MicroAmp Fast Optical 96-Well Reaction Plates were analyzed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using cycling conditions per manufacturer's instructions. *Actb* (Mm01205647_g1) was used as the reference gene. Data was collected and relative fold change compared to acetone (vehicle control) was calculated using the cycle threshold (Ct) and the $2^{-\Delta\Delta Ct}$ method. Genes evaluated include *Flg* (Mm01716522_m1), *Flg2* (Mm02744902_g1), *Ivl* (Mm00515219_s1), *Lor* (Mm01962650_s1), *Krt10* (Mm03009921_m1), *Krt14* (Mm00516876_m1), tight junction protein 1 (*Tjp1*) (Mm00493699_m1), occludin/ELL domain containing 1 (*Ocel1*) (Mm01349279_m1), integrin subunit beta like 1 (*Itgbl1*) (Mm01200043_m1), *S100a8* (Mm00496696_g1), *Tslp*

(Mm01157588_m1), e-cadherin (*Cdh1*) (Mm01247357_m1), Toll-like receptor 4 (*Tlr4*) (Mm00445273_m1), interleukin 4 (*Il4*) (Mm00445259_m1), and interleukin 22 (*Il22*) (Mm00444241_m1). For *Il4* and *Il22*, the Ct was undetected in some vehicle control samples, and for these undetected samples the Ct was set at 40 to calculate fold change.

Bacterial Collection and Isolation

To collect commensal skin bacteria, a sterile foam tipped applicator (Puritan) was moistened with sterile 1X DPBS (Mediatech, Inc.) and then used to swab the dorsal skin of the mouse. Mice were swabbed prior to exposure (day 0), throughout exposure (day 1 and 3), one-day after the last exposure (day 7), and one-week after the last exposure (day 13). The swab was placed into a sterile 2.0 mL Safe-Lock Eppendorf tube and stored at -80 °C until processed. Microbial DNA was isolated as previously described (Meisel *et al.*, 2016), with several modifications. Briefly, cells were lysed by adding 300 µL Yeast Cell Lysis Solution from the MasterPure Yeast DNA Purification Kit (Epicentre) and 0.5 µL Ready-Lyse Lysozyme Solution (Epicentre) to each sample. Samples were incubated, mechanically lysed using 0.5 mm glass bead tubes (Qiagen), and then incubated a second time as previously described. Samples were placed on ice for 5 minutes. Protein was precipitated out of samples using 150 µL Protein Precipitation Reagent from the MasterPure Yeast DNA Purification Kit (Epicentre) and samples were vortexed for 10 seconds. Samples were centrifuged at maximum speed for 10 minutes, supernatant was transferred to a new tube, and 500 µL isopropanol (CAS# 67-63-0) was added to supernatant. Samples were transferred to PureLink Genomic DNA columns (Invitrogen) and centrifuged. Purification steps were followed as per manufacturer's instructions, with 50 µL elution volume. To collect gut bacteria, fecal pellets were collected into sterile 2.0 mL Safe-Lock tubes (Eppendorf) following euthanasia and stored at -80 °C until processed. Fecal matter was weighed and recorded prior to isolation. Gut

microbial DNA was isolated from the fecal matter using the DNeasy PowerSoil Kit (Qiagen) as per manufacturer's instructions, with the following exception. Samples were vortexed vertically on a vortex mixer for 15 minutes. DNA was eluted with 100 µL of the elution buffer and frozen at -80 °C until sequenced. DNA concentration was determined using a Qubit 2.0 Fluorometer (Invitrogen) and DNA quality was checked on agarose gels.

Microbiota Sequencing

For the skin microbiota, the library was constructed using the 16S MetaVx Library Preparation (GENEWIZ, Inc.). Amplicons were constructed with 50 ng DNA or maximum volume for samples with low quantity. The bacterial 16S ribosomal RNA gene V3-V4 region was amplified using forward primers containing the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence "GGACTACNVGGGTWTCTAATCC". For the gut microbiota, the V3-V4 region was amplified using forward primers containing the sequence "CCTACGGGNGGCWGCAG" and reverse primers containing the sequence "GACTACHVGGGTATCTAATCC." Samples had unique barcodes for identification. Indexes/adaptors were added to the ends by PCR. Libraries were validated with Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified using Qubit 2.0 Fluorometer or qPCR. DNA libraries were run on an Illumina MiSeq. Sequencing was performed with a 2 x 250 paired-end configuration.

Microbiome Sequencing Analysis

Paired-end sequencing reads were demultiplexed and analyzed by QIIME2 (Quantitative Insights Into Microbial Ecology) (Bolyen *et al.*, 2019). Briefly, DADA2 (Callahan *et al.*, 2016) was used to trim, denoise, and join to generate counts on unique sequences (termed as features) in each sample. Taxonomy assignment of the features was done with a naïve Bayesian classifier against

SILVA 132 database (Quast *et al.*, 2013) at 99% similarity. For phylogenetic diversity analyses, features assigned to the kingdom Bacteria were used and singleton features were removed. Alpha (Shannon's diversity index) and beta diversity metrics (weighted UniFrac distance) were computed and principal coordinates analysis (PCoA) plots using Emperor (Vázquez-Baeza *et al.*, 2013) were generated for each of the beta diversity metrics.

Statistical Analysis

One-way analysis of variance (ANOVA) was conducted for all experiments containing 3 or more groups followed by a Dunnett's Multiple Comparison Test. For TEWL and skin microbiome analysis, a repeated measures ANOVA was conducted followed by a Dunnett's Multiple Comparison Test compared to day 0. Unpaired t-tests were conducted for experiments with two groups. Mann-Whitney test was conducted for KRT14 protein analysis due to unequal variance. Statistical analysis was conducted using GraphPad Prism (v. 5.0). All differences were considered significant at $p < 0.05$.

References

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