Materials and Methods

Tissues

EpiDerm^{TM} (MatTek) tissues were equilibrated per manufacturer's instructions. Briefly, tissues were placed at 4 °C for 2 hours upon arrival, then transferred to 6-well plates containing 0.9 mL of pre-warmed Dulbecco's Modified Eagle's Medium (hydrocortisone free) containing epidermal growth factor, insulin, other proprietary stimulators of epidermal differentiation, gentamicin (5 µg/mL), amphotericin B (0.25 µg/ml), phenol red, and proprietary lipid precursors (MatTek) /well. Tissues were incubated at 37 °C with 5% CO_2 overnight to equilibrate. The following morning, tissues were washed with 100 µL pre-warmed DPBS and either (1) media was replaced with 0.9 mL fresh pre-warmed media/well (5-day experiments) or (2) tissues were transferred to 12-well hanging-top lids with plates containing 5 mL of pre-warmed media/well (6h, 24h, and 48h experiments). For the 5-day experiment, media was replaced daily throughout the experiment, prior to each exposure.

Triclosan Exposures

Triclosan (CAS # 3380-34-5) was purchased from EMD Millipore Corp. Acetone was selected as the vehicle based on solubility and previous use in evaluating triclosan exposure on EpiDerm tissues (Marshall et al. 2015). Acetone (CAS # 67-41-1) was purchased from Sigma-Aldrich. EpiDerm (3/group) were exposed on the apical side to 30 µL acetone (vehicle) or triclosan (0.05-0.2%) dissolved in acetone (w/v) once for 6, 24, or 48 hours or once/day for 5 consecutive days. Experiments were independently performed twice for each timepoint and endpoint. An additional experiment was performed to compare no exposure vs. acetone control. Tissues were incubated at 37 °C with 5% CO_2 during the exposure. The concentrations were selected based on

a previous toxicity assessment of EpiDerm tissues to triclosan where ≥ 0.188% triclosan was determined to be toxic (Marshall et al. 2015).

LDH Release Assay

Culture media (10 µL) was collected after 6, 24, or 48 hours of the single exposure or each day of the 5-day exposure and transferred to a clear flat-bottomed 96-well plate. The LDH-Cytotoxicity Colorimetric Assay Kit II (BioVision) was used to perform the lactate dehydrogenase (LDH) release assay per manufacturer's instructions. Absorbance (450 nm) was measured using a µQuant (BioTek) or Varioskan Lux (Thermo Scientific) microplate spectrophotometer in duplicate for each sample. Values were averaged and fold change compared to vehicle control was calculated.

Gene Expression Analysis

EpiDerm tissues were washed with 100 µL pre-warmed DPBS immediately prior to tissue collection. EpiDerm tissues were disrupted and homogenized in 700 µL QIAzol using a steel bead and TissueLyser II or with a pellet pestle homogenizer. Homogenates were centrifuged at 15,000 g for 10 minutes at 4 °C. Total RNA was isolated from lysates using the miRNAeasy kit (Qiagen) per manufacturer's instructions. Elution volume was 30 µL per sample. The RNA purity and yield were determined on a NanoDrop Spectrophotometer. 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 was performed per manufacturer's instructions. Plates were analyzed on a 7500 Fast Real-Time PCR System (Applied Biosystems), StepOnePlus (Applied Biosystems), or QuantStudio3 (Applied Biosystems) using cycling conditions per manufacturer's instructions. GAPDH

(Hs02786624_g1) was used as the reference gene. Data was collected and relative fold change compared to vehicle was calculated using the cycle threshold (Ct) and the $2^{-\Delta\Delta Ct}$ method. Data from one tissue insert (5-day exposure) was identified to be an outlier (Grubbs' test) and excluded from analysis. Genes evaluated include filaggrin (FLG) (Hs00856927_g1), FLG2 (Hs00418578_m1), involucrin (IVL) (Hs00846307_s1), loricrin (LOR) (Hs01894962_s1), tight junction protein 1 (TJP1) (Hs01551861_m1), occludin (OCLN) (Hs00170162_m1), keratin (KRT) 10 (KRT10) (Hs00166289_m1), KRT14 (Hs00265033_m1), e-cadherin (CDH1) (Hs01023895_m1), TSLP (Hs00263639_m1), S100A8 (Hs00374264_g1), IL-1A (Hs00174092_m1), IL-1B (Hs01555410_m1), tumor necrosis factor (TNF) (Hs00174128_m1), CXCL1 (Hs00236937_m1), CXCL2 (Hs00169233_m1), and CXCL8 (Hs00174103_m1).

Cytokine Release

Culture media was collected and frozen at -80 °C. Media was evaluated with a custom human premixed multi-analyte kit (LXSAHM; R&D Systems) and analyzed on a MagPix (Luminex) per manufacturer's instructions. Analytes measured: epidermal growth factor (EGF), IL-1α, IL-6, IL-18, IL-33, S100A8, TNF alpha (TNF-α), vascular endothelial growth factor (VEGF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-8, IL-31, IL-36β, transforming growth factor alpha (TGF-α), TSLP.

Permeability Assay

EpiDerm tissues were washed with 100 µL pre-warmed DPBS immediately prior to the permeability assay. Tissues were exposed on the apical side to 40 µL of 1 mM Lucifer Yellow CH dilithium salt (Sigma) dissolved in DPBS and incubated at 37 °C with 5% CO_2 for 2 hours. Media (100 µL/replicate) was collected, and fluorescence intensity was measured in triplicate in 96-well plates read on a Synergy (BioTek) or Varioskan Lux (Thermo Scientific) microplate reader

(excitation 428 nm, emission 536 nm). Fluorescence intensity was averaged per sample and fold change in fluorescence intensity compared to vehicle control was calculated.

References

Marshall NB, Lukomska E, Long CM, Kashon ML, Sharpnack DD, Nayak AP, Anderson KL, Jean Meade B, Anderson SE. 2015. Triclosan induces thymic stromal lymphopoietin in skin promoting th2 allergic responses. Toxicol Sci. 147(1):127-139.