

MATERIALS AND METHODS

Animals – Female BALB/cAnNTac (BALB/c) mice were used in these studies. This strain has a T_H2 bias and is commonly used for investigation of allergic disease and has previously been used to investigate the immunological effects of triclosan. The mice were ordered from Taconic at 6-8 weeks of age and housed 3-5 per cage in ventilated plastic shoebox cages with hardwood ship bedding. Upon arrival, the mice were allowed to acclimate for at least 5 days. Each shipment of animals was randomly assigned to a treatment group. Animals were fed NIH-31 modified 6% irradiated rodent diet with tap water provided from water bottles. A light/dark cycle was maintained on 12-hour intervals. Animals were euthanized by CO₂ asphyxiation. All animal experiments were performed in the AAALAC International accredited NIOSH animal facility in accordance with an animal protocol approved by the CDC-Morgantown Institutional Animal Care and Use Committee.

Triclosan exposures – The concentrations of triclosan (Calbiochem; CAS #: 3380-34-5) used in these experiments were determined to be non-toxic and based on findings from previous studies. The selected doses also represent translationally-relevant exposure conditions, as triclosan concentrations in products have been reported to range between 0.03 and 1.0%. For all studies, BALB/c mice (5/group) were topically treated with acetone vehicle or increasing concentrations of triclosan (0-3%) on the dorsal surface of each ear (25µl/ear) once a day for 1, 2, or 4 consecutive days. Animals were euthanized by CO₂ asphyxiation 24 hrs after the last exposure.

Tissue Collection and Processing - Following euthanasia, ears and right and left auricular draining lymph nodes (dLNs) were used for subsequent analyses. dLNs were collected in 2 ml sterile phosphate-buffer saline (PBS) (pH 7.4) and single cell suspensions (2 nodes/animal) were prepared by mechanical disruption of tissues between frosted microscope slides. Ears (2 per

mouse; split into ventral and dorsal halves) were collected in RPMI and either used intact or processed into single cell suspensions prepared by incubating with a 0.25 mg/ml Liberase-TL Research grade (Roche) enzymatic digestion for 90 min at 37°C in RMPI with 100 µg/ml DNase

I. Skin tissue was further homogenized using a gentleMACS dissociator (Miltenyi) and passed through a 70 µm cell strainer. Tissue viability and total cellularity was assessed using a Cellometer (Nexcelom) based on acridine orange and propidium iodide (AO/PI) incorporation.

Caspase-1 inflammasome activation – Ear and dLN caspase-1 activation was assayed via Promega Caspase-Glo 1 Inflammasome kit according to manufacturer's instructions. Ears (intact; split into dorsal and ventral halves) were incubated for 1.5 hrs in complete EMEM at 37°C/5% CO₂. Following the 1.5 hr incubation supernatants were collected. Following mechanical disruption, the dLN cells were plated at 50,000 cells/well. Caspase-Glo reagent was prepared according to manufacturer's instructions and added to the plate containing the cells/supernatant. Luminescence was read via plate reader after a 2 hr incubation at room temperature. Background luminescence was subtracted and normalized to the corresponding control on each day of the experiment.

Gene expression analysis - Animals were euthanized by CO₂ inhalation 24 hrs after the final exposure. Left and right ears and left and right auricular dLNs were collected into tubes containing 500 µL RNAlater (Invitrogen) and samples were frozen at -80 °C until processed. Tissues were thawed and homogenized with TissueLyser II. Total RNA was isolated from the ear (RNeasy kit) and dLNs (miRNeasy kit) according to the manufacturer's directions (Qiagen). A QIAcube (Qiagen) automated RNA isolation machine was used in conjunction with the specified RNA isolation kit. The concentration and purity of the isolated RNA was determined using a NanoDrop Spectrophotometer (Thermo Scientific). The cDNA synthesis (2000 ng) was

performed using Life Technologies high capacity cDNA reverse transcription kit according to manufacturer's recommendations. For analysis of mRNA expression, a reaction of cDNA, TaqMan mouse specific mRNA primers (Life Technologies), and TaqMan Fast PCR Master Mix (Life Technologies) were added to a MicroAmp Fast Optical 96-well Reaction Plate and analyzed on an Applied Biosystems 7500 Fast Real Time PCR System using default cycling conditions for comparative CT analysis. The mRNA β -actin was used as the endogenous reference. Data were collected and expressed as relative fold change over vehicle control using the $2^{-\Delta\Delta C_t}$ method.

IL-1 β protein assessment – Acetone and triclosan exposed ears and dLNs from BALB/c mice were collected into RPMI and PBS, respectively, following euthanasia. The ears were split into ventral and dorsal halves and incubated intact for 6 hrs in complete EMEM at 37°C/5% CO₂ to allow time for IL-1 β to be secreted. Following the 6 hr incubation, supernatants were collected. After mechanical disruption of dLNs, cells were centrifuged, and supernatants were collected. Supernatants from both skin and dLNs were used for IL-1 β ELISA according to manufacturer's instructions using Mouse IL-1 β Quantikine ELISA Kit (R&D Systems).

Extracellular ATP assay – ATP production was assayed via Promega ToxGlo kit. Acetone and triclosan exposed ears from BALB/c mice were excised and separated into ventral and dorsal halves. Both halves were incubated intact for 1.5 hrs in complete EMEM at 37°C/5% CO₂ to allow time for IL-1 β to be secreted. After mechanical disruption of dLNs, cells were centrifuged, and supernatants were collected. Supernatants from both skin and dLNs were used to measure extracellular ATP. Luminescence was read via plate reader, background readings were subtracted and normalized to corresponding control on each day of the experiment.

Flow cytometry – For phenotypic analysis, single cell suspensions of ears and dLNs were resuspended in FACS buffer containing α -mouse CD16/32 antibody (Fc Block) (BD Bioscience) then incubated with a staining cocktail of fluorochrome-conjugated antibodies specific for mouse cell surface epitopes: CD45-BV605 (clone 30-F11), CD11b-PerCP-Cy5.5 (clone M1/70), CD3-PE-CF594 (clone 145-2C11), Ly6G-FITC (clone 1A8) (BD Biosciences); Ly6C-BV421 (HK1.4) (Biolegend); CD169-eF660 (SER-4) (eBiosciences). Cells were then washed, fixed in Cytofix buffer (BD Biosciences) and resuspended in FACS buffer. Events ($\sim 1\text{--}2 \times 10^6$ /sample) were collected on an LSR II flow cytometer (BD Biosciences) and analyzed using Flowjo software v10.

Mitochondrial mass and mitochondrial reactive oxygen species - For staining of mitochondrial mass, single cell suspensions of dLN were resuspended in 100 μ l of 200 nM MitoTracker Green dye for 20 mins at 37°C/5% CO₂. For mitochondrial ROS, single cell suspensions of dLN were resuspended in 100 μ l of 5 μ M MitoSOX Red dye for 20 mins at 37°C/5% CO₂. Cells were washed with PBS, filtered through a 70- μ m cell strainer, and resuspended in buffer with 50 μ g/ml DNase. Events ($\sim 80,000 - 150,000$ /sample) were collected on an LSR II flow cytometer (BD Biosciences) and analyzed using Flowjo software v10. All cells were gated on single events followed by lymphocyte gating and lastly gated on either MitoTracker⁺ or MitoSOX⁺ cells.

Mitochondrial Membrane Potential – Mitochondrial membrane potential was measured via MitoProbe JC-1 Assay Kit (ThermoFisher Scientific) according to manufacturer's instructions. Briefly, dLN single cell suspensions were diluted to 1×10^6 cells/ml with a final concentration of 2 μ M JC-1 dye and incubated for 15 mins at 37°C/5% CO₂. CCCP was used as a positive control. After 15 mins, cells were washed and resuspended in staining buffer. Events

(~30,000/sample) were collected on an LSR II flow cytometer using FITC and PE channels of a 488 laser and manually compensated and analyzed using Flowjo software v10. Median fluorescence intensity was measured and normalized to that day's corresponding control.

Transmission electron microscopy (TEM) – After single cell preparation, ~750,00 dLN cells/sample were fixed with Karnovsky's fixative, post-fixed with osmium tetroxide, mordanted in tannic acid, stained with uranyl acetate, alcohol dehydrated, and embedded in Epon. The blocks were sectioned and stained with uranyl acetate and lead citrate. Ultrastructural changes were evaluated using a JEOL JEM-1400Plus transmission electron microscope (JEOL, Tokyo, Japan). Morphometric analyses were performed using NIH Fiji ImageJ with at least 45-60 mitochondria (from at least 10 cells) per concentration. Mitochondria with clearly discernible outlines were manually traced on TEM images. Aspect ratio (AR) is defined as [major axis/minor axis]. Circularity [$4\pi \times (\text{surface area}/\text{perimeter}^2)$] and roundness [$4 \times (\text{surface area})/(\pi \times \text{major axis}^2)$] are measurement for sphericity where 1 is a perfect sphere. Percent mitochondria that show standard mitochondrial morphology and altered morphology were assessed by defining altered mitochondria as having a fragmented crista, inhomogeneous electron transparent matrix, and/or U-, C-, or ring-shaped morphology.

Whole cell protein lysates – Ears and dLN were collected in 750 μL (ears) and 350 μL (dLN) T-protein extraction reagent (PER) buffer containing protease and phosphatase inhibitor cocktail and EDTA following euthanasia and mechanically disrupted using TissueLyser II in T-PER with protease and phosphatase inhibitor cocktail. The soluble protein fraction was collected and quantified by BCA protein assay (Pierce), according to the manufacturer's instructions.

Capillary western immunoassay – The capillary western immunoassay experiments were performed according to the ProteinSimple Wes user guide for a 12-230 kDa Separation Module

and the Anti-Rabbit detection module. Briefly, prepared skin and dLN lysate samples were diluted to an appropriate concentration in sample buffer then mixed together with fluorescent master mix and incubated at 95°C for 5 mins. Appropriate sample concentrations were determined by linear range finding as suggested by the manufacturer, where a range of 0 to 1 mg/mL of sample was run to determine the lower (limit of detection) and upper (total saturation) detection limits of the assay with a specific antibody. The signal should change in direct proportion to the amount of protein in the sample. Sample concentrations and antibody dilutions are shown in Table 1. Experiments were performed in the Wes instrument. Instrument settings used were: stacking and separation at 350V or 375 V for 25-31 min; blocking reagent for 30 min, primary and secondary antibody both for 30 min; luminol/peroxide chemiluminescence detection for ~15 mins (exposures of 1-2-4-8-16-32-64-128-512s). The results were inspected to check the automatic peak detection and identification of specific peaks. Criteria used were a peak signal-to-noise (S/N) ratio above 10 and the peak height/baseline ratio above 3. Area under the curve (AUC) was determined via Compass Software for Simple Western (ProteinSimple). Total protein was used as a loading control and for normalization.

Table 1: Primary and secondary antibodies used for ProteinSimple Wes Immunoassay

Antibody	Reference	Sample Concentration	Antibody Dilution	Secondary antibody
Opa1	Novus Biologicals NB-110-55290SS	0.2 mg/ml (skin)	1:200 (skin)	Anti-rabbit-HRP (Wes detection kit)
		0.2 mg/ml (dLN)	1:200 (dLN)	
Drp1	Novus Biologicals NB110-55288SS	0.1 mg/ml (skin)	1:150 (skin)	Anti-rabbit-HRP (Wes detection kit)
		0.1 mg/ml (dLN)	1:150 (dLN)	