Chemical exposures

Mice were dosed daily with acetone (VC: vehicle control) (Sigma-Aldrich; CAS # 67-64-1) or 2% triclosan (TCS) (Calbiochem; CAS #: 3380-34-5) diluted in acetone, starting at study day -7 through 10 days post infection (dpi). Chemicals were applied on the dorsal surface of each ear in a 25 µl/ear volume. Timing and concentration of TCS dosing was determined based off of previous studies for augmenting Th2 immune responses.

Animals

Female BALB/c mice (6-8 weeks-of age) were purchased from Taconic. This strain has previously been used in our laboratory to thoroughly characterize immune responses following topical exposure to TCS. Each shipment of animals was randomly assigned to a treatment group, weighed and individually identified (via tail marking) using a permanent marker and acclimated for a minimum of five days upon arrival. The animals were housed at a maximum of five per cage in ventilated plastic shoebox cages with hardwood chip bedding. NIH-31 modified 6% irradiated rodent diet (Harlan Teklad) and tap water were provided from water bottles, *ad libitum*. The animal facility temperature was maintained at 68–72ºF and the relative humidity between 36–57%. A light/dark cycle was maintained on 12-hour intervals. 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.

Intranasal influenza infections

Mouse-adapted A(H1N1) influenza A/Puerto Rico/8/34 (PR8) was generously provided by Dr. Gary Burleson (Burleson Research Technologies, Inc.) and propagated in the allantoic cavity of

SPF Research Grade Fertile Chicken Egg Flock R128 (Charles River). A dose finding study (30 mice over a 14-day infection period) was performed to determine a sub-lethal dose (50 plaque forming units (pfu)) that was used in these studies. Following infection, mice were monitored for weight-loss and other signs of morbidity (e.g., hunched posture, respiratory stress, or other abnormal behaviors) throughout the course of infection at least every 24 hours; mice were monitored every 12 hours between once weight loss was recorded and symptoms started to be observed. Mice were euthanized if weight loss exceeded 20% and mice showed signs of respiratory stress. In the range finding experiment 8 mice met this criteria for euthanasia, and were humanely euthanized prior to the end of the study by injection of a sodium pentobarbital-based solution. For infections, virus was diluted in USP phosphate buffered saline (S) in order to obtain a dosage of 50 pfu/50 µl. Virus or S was instilled intranasally in mice that were anesthetized with isoflurane. Mice were monitored following infection for recovery from anesthesia and infection as described above. The combined chemical exposures and influenza infections resulted in a total of 40 mice in four treatment groups per timepoint (10 days of infection and 40 days of infection) (Acetone + S [VC/S], triclosan + S [TCS/S], Acetone + PR8 [VC/PR8] and triclosan + PR8 [TCS/PR8]).

Fig 1. Diagram of experimental scheme. BALB/c mice were dermally dosed with acetone or 2% TCS on the dorsal surface of the ear (25 µl/ear) starting at day -7 and continuing through 10 days post infection (dpi). On Day 0, mice were intranasally inoculated with 50 pfu of Influenza A virus PR8 (PR8) diluted in saline or mock infected with saline in a 50 µl volume. Mice were sacrificed on days 1 and 3 post infection to assess viral titers and at days 10 and 40 post infection to assess adaptive immune responses as noted. Exposure groups contained 5 mice/group per timepoint.

Tissue collection and processing

Animals were humanely euthanized by injection of a sodium pentobarbital-based solution on the indicated dpi. Lungs collected for plaque assays were excised and placed in a sterile 2 ml snap cap tube and immediately frozen in liquid nitrogen. Snap-frozen tissues were stored at -80 ºC until used. Cells and proteins from the lung airways were obtained by means of bronchoalveolar lavage (BAL) in which the trachea was intubated, and 1 ml S was introduced and recovered from the airways. The first fraction of BAL was kept separate, while the 2nd and 3rd washes were combined. The first BAL fraction was centrifuged to pellet cells (5 minutes at ~750 x g) and the supernatant (BAL fluid; BALF) was collected and stored at -20 ºC to be used for protein analysis. The cellular portion of the first fraction was combined with the others collected to use for cellular phenotyping. Lungs, perfused with S, were collected into RPMI and kept on ice. Lungs were manually chopped with scissors into small pieces and digested at 37 ºC for one hour in a digestion media containing 0.125 mg/ml Liberase TL (Roche) and 50 ug/ml DNase 1 (Sigma) in RPMI. Digestion was stopped by the addition of RPMI/10% FBS. The lung tissue was then mechanically disrupted on a gentleMACS dissociator (Miltenyi Biotech) then filtered through a 70 μm nitex filter to obtain a single cell suspension. Lung associated lymph nodes (LLNs) and spleens were collected into RPMI, then mechanically disrupted between the frosted ends of two microscope slides and filtered through a 70 μm pore filter to obtain a single cell suspension. Cells were centrifuged (5 minutes at ~750 x *g*) and resuspended before counting. LLNs and spleen cells were counted using a coulter counter (Beckman Coulter) following RBC lysis with Zapoglobin lytic reagent (Beckman Coulter). BAL and lung cells were counted on a Cellometer (Nexcelom) using AO/PI solution (Nexcelom) to determine numbers of live cells.

Lung homogenization

For viral plaque assay (VPA) analysis, excised lung tissue was homogenized using a TissueLyser II (Qiagen). Briefly, 50 mg of thawed lung tissue was placed in a 2.0 ml microtube containing 500 µl cold Hank's Balance Salt Solution (HBSS; ThermoFisher Scientific) supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich), 100 units/ml penicillin G and 100 units/ml streptomycin (ThermoFisher Scientific), and one 5 mm stainless steel bead (Qiagen). Sample tubes were loaded into a cold TissueLyser II rack and homogenized at 30 Hz for 3 minutes. Homogenized lung tissue samples were serially diluted in supplemented HBSS and stored on ice until VPA.

Viral plaque assay

Madin-Darby Canine Kidney (MDCK) cells (ATCC) cultured to ~90% confluence were detached with 0.25% Trypsin-EDTA (ThermoFisher Scientific), washed and re-suspended in complete Eagle's Minimum Essential Medium (EMEM; ATCC) at a density of 1.0×10^6 cells/ml. Next, 2.0 ml of the cell suspension was added to each well of a 6-well CoStar tissue culture plate (Corning) and incubated overnight at 35 ^{o}C in a humidified 5% CO_2 incubator. Confluent cell monolayers were washed twice with 2.0 ml of 1X S, inoculated with 400 µl of diluted, homogenized lung tissue and incubated for 45 min at 35 ^{o}C in a humidified 5% CO_2 incubator. Inoculated cells were then washed once with 2.0 ml of 1X S, overlaid with supplemented Dulbecco's modified Eagle's medium (DMEM)/F12 containing 100 units/ml penicillin G/100 µg/ml streptomycin (ThermoFisher Scientific), 2 mM l-glutamine (ThermoFisher Scientific), 0.2% BSA (Sigma–Aldrich), 10 mM HEPES (ThermoFisher Scientific), 0.22% sodium bicarbonate (ThermoFisher Scientific), 0.01% DEAE-dextran (MP BioMedicals, LLC, Solon, OH), 0.6% agarose (Oxoid Ltd.)

and 2 μg/ml N-p-tosyl-l-phenylalanine chloromethylketone (TPCK; Sigma–Aldrich), and incubated at 35 ^{o}C for 52-hours in a humidified 5% CO_2 incubator. The cells were then fixed with 2.0 ml of 10% formalin for 15 minutes and the agarose overlay was removed by washing with tap water. Plaques were stained with 2.0 ml of 1% crystal violet/0.19% methanol for 15 minutes, rinsed with tap water, dried and plaque forming units (PFUs) were calculated.

TCID50/HA assay

Influenza virus titers in mouse lung tissues were determined using the tissue culture infective dose 50 % (TCID50) combined with hemagglutination (HA) assay. Mouse lungs were placed in 2 ml of ice-cold S and homogenized using gentleMACS Dissociator (Miltenyi Biotec GmbH) and centrifuged at 600 g for 10 minutes. Supernatant was collected and used for the TCID/HA assay. Ten-fold serial dilutions of the supernatants were prepared using Minimal Essential Medium (MEM, ATCC) containing 2 μg/ml TPCK. MDCK (1.0×10^5 cells/mL) cells were plated in a U-bottom 96-well plate (Corning). The diluted supernatants were added to the 96 well plate and then incubated overnight at 37 °C/ 5% CO_2 incubator. The next day, the medium was replaced with fresh MEM. After 72 hours of incubation, 50 μl of 0.5% turkey RBCs in S was added to each well. The plates were then incubated for 1 h at 4°C and the HA patterns were read to determine the TCID50. A positive (PR8) and corresponding negative controls were added in each plate. Hazy wells show turkey erythrocytes agglutination while non-agglutinated wells show a distinct RBC “button” at the bottom of the well. TCID50 was then calculated by the Reed-Muench formula.

Gene expression analysis

An aliquot of cells from the single cell preparation of lung tissue was resuspended in Qiazol (Qiagen) and stored at -80 ºC until analysis. RNA was extracted using a RNeasy kit according to

the manufacturer's directions (Qiagen) on an automated RNA isolation machine (QIAcube, Qiagen). The RNA concentration and purity were determined using a NanoDrop Spectrophotometer (Thermo Scientific). The cDNA was prepared on an Eppendorf Mastercycler using Applied Biosystems' High Capacity Reverse Transcription kit. The cDNA was used as template for real-time PCR (RT-PCR) reactions containing TaqMan PCR Master Mix with gene-specific primers (Applied Biosystems) on a 7500 RT-PCR System. Relative fold changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method compared to the VC/S group. Data were normalized for expression of housekeeping gene *Actb*. Genes assessed included: *Ifng, Tbet, Il4, Cxcr3, Foxp3, Lag3, Gata3, Tnf, Ifnar1,* and *Tslp.*

Luminex assay

Cytokines were measured in 50 µl of BAL fluid using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 17-Plex Mouse ProcartaPlex™ Panel (ThermoFisher) according to manufacturer's instructions and data was acquired using a Luminex 200 system (Millipore).

Flow cytometry

The influenza A $HA_{143-155}$ MHC II (I-A(d)/ HNTNGVTAACSHE) tetramer (APC conjugated) was generated at the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University, Atlanta, GA). The influenza A $NP_{147-155}$ MHC I (H-2K(d) /TYQRTRALV) tetramer (PE conjugated) was purchased from MBL International (Woburn, MA). Due to constraints on cell numbers flow cytometry was only performed on LLNs collected from infected mice. Single cell suspensions were spun down and resuspended in 50 µl of RPMI + 5% FBS containing FC Block (anti-mouse CD16/32 antibody [BD Biosciences]) and 20 µg/ml of the influenza A $HA_{143-155}$ MHC II (I-A(d)/ HNTNGVTAACSHE) tetramer (CD4-Tet) and incubated at 37 ºC for 1 hour. Following

the incubation, a cocktail containing the Influenza A $NP_{147-155}$ MHC I (H-2K(d)/TYQRTRALV) tetramer (CD8-Tet) and remaining cellular surface stains in FACS buffer was added (50 µl/sample) and cells were stained for an additional 30 minutes at RT. The fluorochrome-conjugated anti-mouse antibodies included in the remaining surface-staining cocktail were: CD8-Tet-PE, CD4-BV605 (GK1.5, Biolegend), CD8-V500 (53-6.7, BD Bioscience), CD45-eF450 (30-F11), and CD44-APC-eF780 (IM7). Following surface staining, the cells were washed and intracellular staining was carried out using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience/ThermoFisher) according to manufacturer's instructions. The fluorochrome conjugated antibodies used to stain transcription factors were purchased from Invitrogen: GATA3-FITC (TWAJ) and T-bet-PE-Cy7 (eBio4B10). Appropriate staining controls were included for all tissues including FMO controls, and a human CLIP peptide control (I-A(d) PVSKMRMATPLLMQA) tetramer (APC conjugated) for the CD4-Tet staining (National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University, Atlanta, GA). Following intracellular staining cells were analyzed immediately on a BD LSRII Flow Cytometer. Flow cytometry data was analyzed using FlowJo software (V10.6.1), live cell and doublet discrimination was performed using FSC-A and SSC-A/H parameters, and FMO controls were used for gating. For the CD4-Tet staining, additional controls used for gating were the Human CLIP peptide control and uninfected animals. For the CD8-Tet staining, uninfected animal controls were used in addition to the FMO control to ensure proper gating.

Statistical analyses

Statistical analyses were performed using Prism v.5.0 (GraphPad Software). When comparing more than 2 groups, one-way analysis of variance (ANOVA) was conducted followed by a

Dunnett's multiple comparison posttest to compare to the VC/S control. Comparisons between the infected groups were conducted using an unpaired student's t-test. Statistical significance is designated by */#= $p < 0.05$, **/## = $p < 0.01$ and ***/### = $p < 0.001$.